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(54) Title: MUTANT TISSUE FACTOR LACKING FACTOR VII ACTIVATION ACTIVITY

(57) Abstract

The present invention describes a mutant human tissue factor protein which binds functional Factor VII/VIIa, proteolytically activates Factor X, but is substantially free of proteolytic activity for the activation of Factor VII, and compositions containing the mutant protein. Also disclosed are diagnostic methods of using the mutant human tissue factor protein for the detection of Factor VIIa, and recombinant DNA vectors for expressing the protein.

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Mutant Tissue Factor Lacking Factor VII Activation Activity

Technical Field

The present invention relates to a functional mutant of human tissue factor (mhuTF) that binds Factor VII or activated Factor VII (VIIa) and can proteolytically convert Factor X into activated Factor X (Xa), but does not significantly proteolytically activate Factor VII. The mutant human tissue factor is useful as a diagnostic and therapeutic reagent.

Background

Initiation of the coagulation cascades *in vivo* is mediated by the cell surface expression of tissue factor (TF) (Davie et al., Biochemistry 30:10363-10370, 1991) which serves as the receptor and catalytic cofactor for the serine protease Factor VIIa (VIIa) as well as a mediator for the auto-activation of VII to VIIa (Nakagaki et al., Biochemistry 30:10819-10824, 1991). Upon assembly with TF, VIIa exhibits enhanced catalytic activity evidenced by hydrolysis of small peptidyl (Ruf et al., J. Biol. Chem., 266:2158-2166, 1991; and Lawson et al., J. Biol. Chem., 267:4834-4843, 1992) as well as protein substrates (Ruf et al., J. Biol. Chem., 266:2158-2166, 1991; and Silverberg et al., J. Biol. Chem., 252:8481-8488, 1977; Osterud et al., Proc. Natl. Acad. Sci. USA, 74:5260-5264, 1977; Bom et al., Biochem. J. 265:327-336, 1990; and Lawson et al., J. Biol. Chem. 266:11317-11327, 1991). Cleavage of small peptidyl substrates is efficient at μM Ca^{2+}, whereas the

activation of macromolecular substrates such as the zymogen Factor X (X) require the presence of Ca^{2+} at concentrations consistent with saturation of the γ-carboxylated amino-terminal domain of VIIa (Ruf et al., J. Biol. Chem., 266:15719-15725, 1991).

The circulating levels of plasma Factor VIIa have been identified as a significant risk factor for myocardial infarction. Meade et al., Lancet, 1:1050, 1980. Therefore, assays to determine the levels of Factor VIIa, rather than Factor VII, are an important supplement to diagnosis.

Accurate determination of plasma VIIa has been questionable due to the rapid conversion of Factor VII to Factor VIIa in most clotting assays.

The assays for Factor VIIa are typically a clotting assay in which patient plasma sample is added to a Factor VII deficient plasma. The addition of a thromboplastin (as a source of wild type tissue factor) and calcium drive the clotting reaction. Thereafter, a standard curve of clotting time versus "percent factor VIIa" is constructed using pooled normal plasma, whereupon patient values are interpolated from the curve. Examples of typical Factor VIIa assays are described by Miller et al., British J. Haematol., 59:249-258, 1985; van Deijk et al., Haemostasis, 13:192-197, 1983: Hoffman et al., J. Clin. Lab. Med., 111:475-481, 1988; Kitchen et al., Thrombosis Res., 50:191-200, 1988; and Seligsohn et al., Blood, 52:978, 1978.

The conventional assays have a number of significant limitations. First, the performance of a clotting assay with standard thromboplastin is not only dependent upon Factor VIIa, but is also

influenced by the concentration of all other vitamin K-dependent clotting factors that might participate in the clotting assay that are present in the Factor VIIa deficient plasma. Second, although clotting in a standard thromboplastin assay is initiated by the patient's VIIa in a sample, additional VIIa will be generated during the assay as Factor Xa is produced which subsequently activates any Factor VII present in the sample. Third, the slope of standard curves for routine assay of Factor VIIa is very shallow, leading to poor precision, because of the performance characteristics of standard thromboplastin. Additional precision difficulties are attendant with the use of "standard" sources of VIIa and thromboplastin, which are highly variable.

There continues to be a need for a simple assay for measurement of Factor VIIa in plasma without the complications of previous assay methods.

Brief Description of the Invention

It has now been discovered that human tissue factor huTF contains sites defined by amino acid residues which, when modified, can alter the function of huTF to selectively inactivate the Factor VII activation activity of the huTF:VIIa complex without affecting the ability of the modified huTF to bind Factor VII or VIIa or the ability of modified huTF:VIIa complex to activate Factor X. The modified (mutant) human tissue factor is referred to as mhuTF.

A region of huTF around the structural loop defined by amino acid residues 157-167 (SEQ ID NO 2) is shown herein to not be required for high affinity binding to Factor VIIa, whereas amino acid residues in

the region are important for the proteolytic activation of Factor VII to VIIa. Therefore, mutations in one or more of the residues in the region of huTF defined by the amino acid residues 106 to 219 shown in SEQ ID NO 2 are desirable for the introduction of the functional defect defined herein.

The selective elimination of the ability of huTF to activate Factor VII is advantageous for use in clinical hospital clotting assays, and in particular for assays to measure the amount of activated factor VII (VIIa) present in a patient, such as for screening of patients at risk for cardiovascular disease.

The mutant huTF described herein provides particular advantages for use as a thromboplastin reagent. These advantages include the ready use in a standard coagulation assay without the complications of the other vitamin K-dependent proteins typically present because the assay biochemistry relies on the typical capacity of the mhuTF:Factor VIIa complex to activate Factor X without "feedback" interference by the production of Factor VIIa during the assay. The selective loss of Factor VII activation allows precision measurement in a standard curve over at least 4 logs of Factor VIIa concentrations.

The mhuTF may be phospholipid reconstituted as described herein for use as a standard thromboplastin reagent, and may readily be adapted for use in standard analytical equipment.

Thus, in one embodiment, the invention describes a mutant human tissue factor protein (mhuTF) having the capacity to bind Factor VII/VIIa and to proteolytically hydrolyze Factor X, but being substantially free of the capacity to activate Factor

VII when present in a complex of mhuTF:VIIa.

A related embodiment describes a composition that comprises a mutant human tissue factor protein of this invention. Preferred compositions further contain liposomes, cryopreservatives, and/or detergents.

In another embodiment, the invention describes a method for detecting the amount of Factor VIIa in a body fluid sample comprising the steps of:

a) admixing a preselected amount of the body fluid sample with a clotting assay admixture, wherein the clotting assay admixture comprises a mutant human tissue factor (mhuTF) composition of this invention and is substantially free of Factor VIIa and wild type human tissue factor, to form a Factor VIIa assay admixture;

b) maintaining the Factor VIIa assay admixture under conditions sufficient for the mhuTF to bind to any of the Factor VIIa present in the sample and form a clot; and

c) determine the amount of time required for the clot to form, which time is proportional to a predefined amount of Factor VIIa, thereby determining the amount of VIIa present in the sample.

Other related embodiments will be apparent based on the disclosures contained herein.

Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

Figure 1 is a schematic representation of residues 151-172 in tissue factor (TF). Alignment to strand C according to Bazan, Proc. Natl. Acad. Sci., USA, 87:6934-6938 (1990) is indicated and the

functionally important residues are highlighted. The single letter code for amino acids is used.

Figure 2 illustrates the VII binding to $TF_{A161D162A163}$. Specific binding of VII to cell surface $TF_{A161D162A163}$ (Figure 2A) and wild-type TF (Figure 2B) is shown. The insets give the Scatchard analysis for the same data obtained in a representative experiment. The binding assay is described in Example 3.

Figure 3 illustrates the amidolytic and proteolytic activity of mutant TF-VIIa complexes. In Figure 3A, the cleavage of small peptidyl substrates was assessed with Spectrozyme FXa in the presence of 10 nM VIIa and 5 nM wild-type or mutant TF. The rate of hydrolysis of the peptidyl substrate was determined in a 200 ul reaction with a kinetic plate reader and is given as the increase in absorbance (mOD/min). In Figure 3B, the activation of Factor X (1 uM) by mutant and wild-type TF in the presence of excess VIIa (5 nM) was determined at 37C. The rate of Xa formation per enzymatic unit TF-VIIa was calculated based on the TF concentration in the assay. Mean and standard deviation calculated for three independent experiments are shown in both panels. The amidolytic and proteolytic assays are described in Example 3.

Figure 4 shows the effect of VIIa on the specific clotting activity of mutant and wild-type TF. Specific functional activity was determined in normal shown in Figure 4A or IX deficient plasma shown in Figure 4B with (hatched bars) or without (filled bars) 500 nM VIIa added. Mean and standard deviation were calculated from three experiments. The functional activity assays were performed as described in Example 3.

Detailed Description of the Invention

A. Definitions

"Amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 C.F.R. 1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine

H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left-to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those listed in 37 CFR 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as NH_2 or acetyl or to a carboxy-terminal group such as COOH.

"Recombinant DNA (rDNA) molecule" refers to a DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e.,

evolutionarily different, are said to be "heterologous".

"Vector" refers to a rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to herein as "expression vectors". Particularly important vectors allow convenient expression of a mhuTF protein of this invention.

"BHT" refers to butyrated hydroxytoluene.

"CHAPS" refers to 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

"MOPS" refers to 3-(N-morpholino)-propanesulfonic acid.

"OTG" refers to octyl beta-D-thioglucopyranoside.

"Phospholipid" refers to an organic molecule derived from either glycerol (most commonly) or sphingosine. Phospholipids derived from glycerol (or phosphoglycerides) comprise a glycerol backbone, two fatty acid chains esterified to the first and second carbons of the glycerol and phosphoric acid esterified to the third carbon. Optionally, an alcohol moiety is esterified to the phosphoric acid.

"PC" refers to phosphatidyl choline, an uncharged phosphoglyceride having an alcohol moiety derived from choline is esterified to the phosphoric acid.

"PE" refers to phosphatidyl ethanolamine, a positively charged phosphoglyceride, having an alcohol moiety derived from ethanolamine is esterified to the

phosphoric acid.

"PG" refers to phosphatidyl glycerol, a negatively charged phosphoglyceride, having an alcohol moiety derived from glycerol is esterified to the phosphoric acid.

"PS" refers to phosphatidyl serine, a negatively charged phosphoglyceride, having an alcohol moiety derived from serine is esterified to the phosphoric acid.

"Prothrombin time" is abbreviated as PT and refers to the time interval between the addition of a thromboplastin or prothrombin time reagent and the appearance of a clot in platelet poor, citrated plasma.

"Prothrombin ratio" is abbreviated as PR and refers to the prothrombin time of an individual's plasma (either normal or abnormal) divided by the prothrombin time of pool of normal individual plasmas.

"rTF" refers to recombinant tissue factor.

"TBS" refers to 20 mM Tris (pH 7.5) containing 150 mM sodium chloride.

B. Mutant Human Tissue Factor

The invention describes a modified (mutant) human tissue factor protein, designated mhuTF, which has the desirable properties of:

(1) high affinity binding to Factor VII or VIIa;

(2) inability to significantly catalyze the proteolytic conversion of VII to VIIa when mhuTF is present in a mhuTF:VIIa complex; and

(3) capacity for hydrolysis of protein substrates such as Factor X and related peptidyl substrates.

By high affinity binding is meant that the mhuTF protein has the ability to bind VII or VIIa at concentrations of VII/VIIa of about 0.1 to 60 nanomolar (nM), preferably 0.5 to 30 nM, and when bound provides a mhuTF:VIIa complex which is capable of hydrolysis of Factor X. When referring to both the active Factor VIIa or inactive Factor VII, the terminology VII/VIIa is used. Representative binding assays for detecting the ability of mhuTF to bind to VII/VIIa are described in Example 3. Factor VII or recombinant VIIa for use in a binding assay are generally available, at least from Novo Nordisk, Inc., (Gentofte, Denmark). Exemplary assays for measuring the capacity for hydrolysis of Factor X are described in Example 3, and can alternatively include the use of Factor X-related peptide substrates such as the chromogenic substrates Spectrozyme FXa, peptide S-2288, and the like, available from American Diagnostica, Inc. (Greenwich, CT).

The proteolytic conversion of VII to VIIa, when mhuTF is present in a mhuTF:VIIa complex, is considered to be insignificant if the inhibition of conversion of VII to VIIa, when measured as described in Example 3, is at least 70%, preferably is at least 90%, and more preferably is about 95-100%, as compared to the rate of conversion by wild type huTF. Stated differently, mhuTF is substantially free of the ability to activate Factor VII when significant activation of Factor VII does not occur in the presence of a mhuTF:VIIa complex. Assays for measuring the proteolytic conversion of Factor VII to VIIa by a mhuTF:VIIA or huTF:VIIa complex are described in Example 3.

Thus, the invention contemplates a mutant human tissue factor protein (mhuTF) having the capacity to bind Factor VII/VIIa and to proteolytically hydrolyze Factor X, but being substantially free of the capacity to activate Factor VII when present in a complex of mhuTF:VIIa.

A preferred mhuTF protein contains one or more mutations in the amino acid residue sequence within the region of huTF at amino acid residues 106 to 219 shown in SEQ ID NO 2 of the mature huTF. The complete amino acid residue sequence of mature wild type huTF is shown in SEQ ID NO 2. Preferred are mutations in the region of residues 147-167 shown in SEQ ID NO 2.

Preferred mutations are substitutions of a native (wild type) amino acid residue for an alternate residue, thereby altering the primary amino acid residue sequence of wild type huTF. Using conventional terminology, a substitution is indicated by listing the substituted residue in single letter code together with the residue position number in the wild type huTF sequence. Thus a mhuTF protein having a substitution of alanine (A) for tyrosine (Y) at residue position number 157 is designated as A^{157}.

Particularly preferred are the mhuTF proteins described herein at Example 3, where specific amino acid substitutions were made in the wild type huTF to produce a modified huTF (mhuTF) having the properties described herein.

Insofar as tissue factor from species other than human are highly related both structurally and in terms of primary sequence, the invention also contemplates mutant tissue factor having the characteristics of mhuTF which are derived from other

mammals, including cow, rat, rabbit, mouse, pig, primates, and the like. The primary amino acid residue sequence of non-human tissue factor is known for a variety of the recited mammalian species, including rabbit, mouse and cow. See, for example, Hartzell et al., Mol. Cell. Biol., 9:2567-2573, 1989; Andrews et al., Gene, 98:265-269, 1991; and Takayeniki et al., Biochem. Biophys. Res. Comm., 181:1145-1150, 1991.

C. DNA Segments and Vectors

In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the structural gene that codes for the protein. Thus, a structural gene or DNA segment can be defined in terms of the amino acid residue sequence, i.e., protein or polypeptide, for which it codes.

An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

The DNA segments of the present invention are characterized as including a DNA sequence that encodes a mutated human tissue factor heavy chain protein (mhuTFh) according to the present invention. That is, the DNA segments of the present invention are characterized by the presence of a mhuTF structural gene. Preferably the gene is present as an uninterrupted linear series of codons where each codon codes for an amino acid residue found in the mhuTFh protein, i.e., a gene free of introns.

One preferred embodiment is a DNA segment that codes an amino acid residue sequence that defines a mhuTF protein corresponding in sequence to a wild type huTF protein except that the amino acid residue sequence has at least one of the substitutions selected from the group consisting of $R^{158}G^{160}$, A^{159}, $R^{158}A^{159}G^{160}$, A^{157}, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$, and the DNA segment is capable of expressing a mhuTF. A preferred DNA segment codes for an amino acid residue sequence consisting essentially of the sequence shown in SEQ ID NO 2 except that the sequence shown contains at least one of the substitutions selected from the group consisting of $R^{158}G^{160}$, A^{159}, $R^{158}A^{159}G^{160}$, A^{157}, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$. Representative and preferred DNA segments are described in the Examples.

Homologous DNA and RNA sequences that encode the above mhuTF are also contemplated.

DNA segments (i.e., synthetic oligonucleotides) that encode mhuTF proteins can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, et al., (J. Am. Chem. Soc., 103:3185-3191, 1981) or using automated

synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define the DNA segment, followed by hybridization and ligation of oligonucleotides to build the complete segment.

Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence.

Furthermore, DNA segments consisting essentially of structural genes encoding a mhuTF protein can be obtained from recombinant DNA molecules containing a gene that defines huTF, and can be subsequently modified, as by site directed mutagenesis, to introduce the desired substitutions.

Site-specific primer-directed mutagenesis is now standard in the art, and is conducted using a primer synthetic oligonucleotide complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage.

Thus, by site-directed mutagenesis, one can readily construct any of the described DNA segments that code a mhuTF described herein, by starting, for example, with the expression vector shown in SEQ ID NO 1 that codes and expresses wild type huTF, and

mutating selected nucleotides, as described herein, to form one or more of the DNA segments that code a mhuTF of this invention.

In addition, the invention contemplates a recombinant DNA molecule (rDNA) containing a DNA segment of this invention. A rDNA can be produced by operatively linking a vector to a DNA segment of the present invention.

As used herein, the term "vector" refers to a DNA molecule capable of autonomous replication in a cell and to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A vector capable of directing the expression of a mhuTF gene is referred to herein as an "expression vector". Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature.

The choice of vector to which a DNA segment of the present invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules. However, a vector contemplated by the present invention is at least capable of directing the replication, and preferably also expression, of the mhuTF structural gene included in DNA segments to which it is operatively linked.

In preferred embodiments, a vector contemplated by the present invention includes a procaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the

recombinant DNA molecule extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, those embodiments that include a procaryotic replicon also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and translation) of the mhuTF gene in a bacterial host cell, such as E. coli, transformed therewith. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10

(Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eucaryotic expression vectors.

In preferred embodiments, the eucaryotic cell expression vectors used to construct the recombinant DNA molecules of the present invention include a selection marker that is effective in an eucaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (neo) gene. Southern et al., J. Mol. Appl. Genet., 1:327-341 (1982). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker. Exemplary is the co-transfection described in the Examples.

The invention also contemplates a host cell transformed with a recombinant DNA molecule of the present invention. The host cell can be either procaryotic or eucaryotic, although eucaryotic cells are preferred. Eucaryotic cells useful for expression of a mhuTF protein are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the mhuTF gene product. Preferred eucaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the

ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eucaryotic tissue culture cell lines. Particularly preferred and exemplary is the CHO-K1 cell line described herein.

Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen et al., Proc. Natl. Acad. Sci. USA, 69:2110 (1972); and Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

With regard to transformation of vertebrate cells with vectors containing rDNAs, see, for example, Graham et al., Virol., 52:456 (1973); Wigler et al., Proc. Natl. Acad. Sci., USA, 76:1373-76 (1979), and the teachings herein.

Successfully transformed cells, i.e., cells that contain a rDNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce monoclonal colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol., 98:503 (1975) or Berent et al., Biotech., 3:208 (1985).

In addition to directly assaying for the presence of rDNA, successful transformation can be confirmed by well known immunological methods when the rDNA is capable of directing the expression of mhuTF, or by

the detection of the biological activity of mhuTF.

For example, cells successfully transformed with an expression vector produce proteins displaying mhuTFh antigenicity or biological activity. Samples of cells suspected of being transformed are harvested and assayed for either mhuTFh biological activity or antigenicity.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. Preferably, the culture also contains a protein displaying mhuTF antigenicity or biologically activity.

Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources. In embodiments wherein the host cell is mammalian, a "serum-free" medium can be used. Preferred is the culturing conditions described herein.

D. Preparation of Mutant huTF

Mutant human tissue factor (mhuTF) of this invention can be produced by a variety of means, and such production means are not to be considered as limiting.

Preparation of a mhuTF typically comprises the steps of: providing a DNA segment that codes a mhuTF protein of this invention; introduction of the provided DNA segment into an expression vector; introduction of the vector into a compatible host cell; culturing the host cell under conditions

sufficient for expression of the mhuTF protein; and harvesting the expressed mhuTF protein from the host cell. In preferred embodiments, the harvested mhuTF is reconstituted into phospholipids as described herein to form a composition containing mhuTF. Exemplary procedures for each of the above-enumerated steps are described in the Examples.

Insofar as the expressed protein is highly related to wild type huTF, the purification of mhuTF can be conducted by a variety of art-recognized procedures for preparing purified huTF from cell culture. See, in particular, the purification procedure described herein.

Thus, in one embodiment, a mhuTF protein is prepared using a DNA segment as described herein. Alternatively, one can use the screening methods described herein to identify additional substitutions of amino acids in the wild type huTF which produce a mhuTF having the disclosed desirable properties. As seen by the numerous mutant constructs described herein, a variety of mhuTF proteins have been designed as produced by the present methods. Additional substitutions (mutations) other than those described specifically herein can be readily designed to form a mhuTF having the disclosed biological activities.

E. Compositions

A mutant human tissue factor protein (mhuTF) of the invention is typically provided in one or more of a variety of compositional forms suitable for the contemplated use. Although mhuTF retains its biological activity in a variety of buffers and solutions, it is preferred to be formulated in a mild

detergent or phospholipid composition. Particularly preferred are phospholipid compositions which afford maximum stability and biological activity of the mhuTF in the composition. Such phospholipid compositions are preferably formulated to form liposome compositions, as are generally well known in the art. Typically, the composition contains an amount of biologically active mhuTF suitable for its contemplated use.

Thus, in one embodiment, the phospholipid composition comprises liposomes having mhuTF associated with the lipid bilayer of the liposomes, such that the mhuTF is inserted through the lipid bilayer.

The lipid bilayer of the liposomes comprises phospholipids, preferably, phosphoglycerides.

Alternatively, according to another aspect of the present invention, mhuTF compositions are provided which comprise phospholipid micelle compositions which have mhuTF associated with phospholipid micelles such that the mhuTF factor is inserted into the micelle.

The mhuTF compositions of the present invention comprise about 0.1 mg to about 3 mg of mhuTF per mg of phospholipid mixture. The ratio of mhuTF to phospholipid mixture may determine the sensitivity of the resulting reagent. Thus, use of a ratio of about 1 to 2 mg mhuTF per mg phospholipid mixture may be suitable for a mhuTF reagent having a International Sensitivity Index ("ISI") of about 1.0. Use of a ratio of about 0.25 to about 0.5 mg mhuTF per mg phospholipid mixture may be suitable to prepare a composition having an ISI of about 1.6 to about 2.0.

Preferred are compositions that additionally

comprise from about 0.5 to about 1.5% (w/v) glycine. Where it is desired to be able to lyophilize the mhuTF composition to allow storage and later reconstitution, the reagent preferably includes a cryopreservative, preferably a carbohydrate preservative, most preferably trehalose.

Suitable phospholipids for use in the liposome compositions of the present invention include those which contain fatty acids having twelve to twenty carbon atoms; said fatty acids may be either saturated or unsaturated. Preferred phospholipids for use according to the present invention include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS). These phospholipids may come from any natural source and the phospholipids, as such, may be comprised of molecules with differing fatty acids. Phospholipid mixtures comprising phospholipids from different sources may be used. For example, PC, PG and PE may be obtained from egg yolk; PS may be obtained from animal brain or spinal chord. These phospholipids may come from synthetic sources as well.

Phospholipid (PL) mixtures having a varied ratio of individual PLs may be used. Suitable PL mixtures comprise (a) from about 20 to about 95 mole percent PC; (b) from about 2.5 to about 50 mole percent PE; (c) from about 2.5 to about 50 mole percent PS; and (d) from about 0 to about 40 mole percent PG. Preferred are PL mixtures comprising from about 5 to 15 mole percent PE, from about 3 to about 20 mole percent PS, from about 10 to about 25 mole percent PG; and the remainder PC, preferably from about 50 to about 90 mole percent PC. Especially preferred are PL

mixtures comprising from about 8 to about 12 mole percent PE, from about 3 to about 10 mole percent PS, from about 14 to about 20 mole percent PG and from about 58 to about 75 mole percent PC.

Although the phospholipids may be used in varied ratios, mixtures of phospholipids having preselected amounts of individual phospholipids result in mhuTF compositions having advantageous activity and stability of activity. Although a wide range of ratios of individual phospholipids may be used, for advantageous activity and stability of the resulting mhuTF composition, a certain level of PS in the total phospholipid composition is preferred. The amount of PS that is preferably present to some extent is determined by the remaining components of the PL mixture and their relative amounts as part of the total PL mixture. For example, use of high amounts of PG, another negatively charged phospholipid, (on the order of about 10% or more) allow use of lower levels of PS, on the order of about 3%. However, if a PL mixture low in PS is used, it is advantageous to include at least about 5% PE preferably at least about 10%.

The phospholipids are conveniently combined in the appropriate ratios to provide the PL mixture for use in preparing the mhuTF composition of the present invention. In one preferred embodiment, the PL mixture may comprise PC, PG, PE and PS in the mole ratio of 67: 16: 10: 7, respectively. In another preferred embodiment, the PL mixture may comprise PC, PG, PE and PS in the mole ratio of 7.5: 0: 1: 1, respectively.

Recombinant mhuTF may prepared by recombinant

technology using methods and expression systems known to the art. See, e.g., Morrissey, J.H., et al., Cell 50: 129-135 (1987); Summers, M.D., "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures," Texas Agricultural Experiment Station, Bulletin 1555 (1987). A preferred method is described herein.

Mutant human tissue factor may also be purified by immuno-affinity chromatography or other chromatographic methods designed to separate a specific protein from other protein contaminants.

Where the mhuTF composition will be lyophilized prior to storage for later use, it is preferred to include a carbohydrate or carbohydrates as cryopreservative(s) to protect the integrity of liposomes in the resulting liposome composition during lyophilization and subsequent rehydration.

Cryopreservation relates to preserving the integrity of delicate substances when liquids containing them are frozen and dehydrated. The use of a carbohydrate as a cryopreservative of liposome integrity upon freezing and subsequent lyophilization has been reported. Racker, E., Membrane Biol., 10: 221-235 (1972); Sreter, F. et al., Biochim. Biophys. Acta., 203: 254-257 (1970); Crowe et al., Biochem. J., 242: 1-10 (1987); Crowe et al., Biochim. Biophys. Acta., 987: 367-384 (1988).

Suitable carbohydrate cryopreservatives include trehalose, maltose, lactose, glucose and mannitol. According to a preferred aspect of the present invention, trehalose is included in aqueous buffer solution used in the preparation of the mhuTF composition of the present invention (prior to

lyophilization), preferably at a concentration in the range of about 50 mM to about 250 mM.

According to a particularly preferred aspect of the present invention, glycine is included as an additional component of a mhuTF composition. Inclusion of glycine in a mhuTF composition results in reagents which exhibit substantially improved performance in PT assays and other assays such as are described herein, giving reproducible biological activity and increased stability. Thus, a preferred mhuTF composition further comprises from about 0.5 percent to about 1.5 percent (w:v) glycine, and more preferably comprises from about 0.6 to about 1.2 percent glycine.

The phospholipids, which may be obtained from the manufacturer in an organic solvent, are mixed together in the appropriate ratios to yield the specified composition. An antioxidant can also be added to reduce alkyl chain peroxidation of the fatty acid portions of the phospholipids, and the organic solvent, if present, is removed by evaporation. One suitable antioxidant is butyrated hydroxy toluene. Preferably about 0.1% (by weight) of antioxidant is used.

The dried (evaporated) phospholipid mixture is then redissolved with an aqueous detergent solution. Suitable detergents include those which have a relatively high critical micelle concentration (CMC). Womack et al., Biochim. Biophys. Acta, 733: 210 (1983). Such detergents include detergents having a CMC of greater than approximately 2 mM. Preferred are those detergents having a CMC of between approximately 2 to 25 mM. Such preferred detergents include

3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and alkylglucopyranosides such as octyl beta-D-glucopyranoside, octyl beta-D-thioglucopyranoside and the like. Optionally, the detergent solution may include other components. These components may include buffer salts such as HEPES, Tris, phosphate, and the like; various other salts such as NaCl, KCl, and the like; a carbohydrate cryopreservative such as trehalose, maltose, glucose, and the like; and glycine.

According to a preferred embodiment of the present invention, the detergent solution comprises 20 mM Tris, pH 7.5, 150 mM NaCl, (TBS) containing 100 mM CHAPS, 150 mM trehalose and 0.8% glycine. According to this preferred embodiment, the phospholipids are redissolved in this solution to give a final concentration of about 20 mg/ml.

Expressed and purified mhuTF, together with carrier protein, are combined with the redissolved phospholipids and the volume of the resulting mixture is adjusted with a buffer as described above, preferably containing cryopreservative (most preferably trehalose) and glycine but no detergent. mhuTF is admixed with carrier protein, such as bovine gamma globulin, and sufficient buffer is added to adjust the final concentrations of tissue factor to 10 mg/ml, bovine gamma globulin to 1 mg/ml, phospholipid to 4 mg/ml and detergent to 20 mM. Suitable buffers include TBS containing 150 mM trehalose and 0.8% glycine.

The resulting clear, colorless solution requires no vortexing or sonicating to ensure co-solubilization.

The detergent in the phospholipid-mhuTF admixture can be removed by a number of methods resulting in a stable liposome composition having mhuTF associated with and inserted through the lipid bilayer. Suitable methods of removal of detergent include dialysis, tangential flow diafiltration, cross flow hollow fiber filtration, treatment with hydrophobic chromatography resin, and simple dilution.

One preferred method of detergent removal from the phospholipid-mhuTF admixture utilizes dialysis for at least 30 hours at room temperature in dialysis membrane tubing against a buffer such as TBS containing 150 mM trehalose, 0.8% glycine and 0.05% NaN_3 to remove the detergent. Another preferred method of detergent removal utilizes resin treatment. Suitable resins include hydrophobic chromatographic resins such as Amberlite XAD-2 (Rohm and Haas Co. in Philadelphia, Pennsylvania) or Bio-Beads SM-2 (BioRad in Richmond, California). The resins may be used to remove the detergent, either by direct contact with the phospholipid-mhuTF solution admixture or separated from it by a dialysis membrane. The rate of removal of detergent from the phospholipid-mhuTF admixture is proportional to the weight ratio of the detergent in solution and the chromatographic resin beads.

The liposome solution resulting from the detergent removal step is then made to 5 mM $CdCl_2$. According to one preferred aspect, the liposome composition which contains the fully active mhuTF is diluted to a concentration 50 mM Tris, pH 7.5, 75 mM trehalose, 0.8% glycine and 10 to 15 mM $CaCl_2$ before use. Alternatively, the diluted reagent may be lyophilized for long term preservation of its

biological performance characteristics and then later reconstituted by suspension in water before use.

Another preferred method of detergent removal avoids the use of either dialysis or resin treatment and yet provides for preparation of active mhuTF reagent. According to this method, detergent solubilized phospholipid compositions containing mhuTF are diluted into a buffer without detergent to produce mixed micelles containing mhuTF which remain capable of being fully activated by $CdCl_2$. According to this aspect of the invention, phospholipids are dissolved to 20 mg/ml in a buffer containing detergent, preferably an alkyl glucopyranoside. A suitable buffer-detergent solution comprises 20 mM HEPES (pH 6) containing 50 mM octyl beta-D-thioglucopyranoside (OTG) and 150 mM NaCl. Carrier protein, mhuTF, and $CdCl_2$ are then added and the mixture diluted further with buffer without detergent, such as 20 mM HEPES (pH 6) containing 150 mM NaCl, to yield final concentrations of mhuTF at 10 mg/ml, carrier protein (bovine gamma globulin) at 1 mg/ml, $CdCl_2$ at 5mM, phospholipids at 4 mg/ml, and OTG at 10 mM. The reagent may be lyophilized for storage as described above, or diluted as described above before use.

According to another aspect of the present invention, this reagent may be prepared by following methods for the preparation of vesicles and detergent-phospholipid mixed micelles from phospholipids by methods based on mechanical means, by removal of organic solvents, by detergent removal, and by size transformation as has been described by Lichtenberg, D. and Barenholz, Y., Methods of Biochemical Analysis, 33: 337-462 (1988), and the

disclosures of which are incorporated herein by reference.

1. Therapeutic Compositions

Insofar as the present invention also contemplates therapeutic uses of a mhuTf protein of this invention, therapeutic compositions useful for practicing the therapeutic methods are also contemplated. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with at least one species of mhuTF as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be

emulsified. Particularly preferred are phospholipid and liposome compositions as described herein. In addition, a therapeutic amount of mhuTF can be present in a ointment or on a diffusible patch, such as a bandage, as to afford local delivery of the agent.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in

addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water, as described herein. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions, particularly the liposome compositions described earlier.

A therapeutic composition contains an effective amount of mhuTF of the present invention, typically an amount of at least 0.1 weight percent of active protein per weight of total therapeutic composition. A weight percent is a ratio by weight of mhuTF protein to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of mhuTF per 100 grams of total composition.

F. Diagnostic Methods

The present invention contemplates various assay methods for analyzing components of the huTF-mediated coagulation cascade through the use of a mhuTF composition of this invention. Particularly preferred are assays for measuring Factor VIIa in a body fluid sample such as plasma or blood.

Those skilled in the art will understand that there are numerous well known clinical diagnostic

coagulation chemistry procedures in which the described mhuTF reagent of this invention can be used. Thus, while exemplary assay methods are described herein, the invention is not so limited.

A preferred assay is the detection of Factor VIIa, as described in detail herein the Examples. The methods generally involves the use of mhuTF in place of huTF in a clotting time assay. The method comprises admixture of the reagents as described in the Examples with a body fluid sample believed to contain VIIa, incubating (maintaining) the admixture for a preselected time under conditions sufficient to allow the clotting reaction to occur, and measuring the amount of time passed, thereby indicating the amount of VIIa present in the sample.

Because a mhuTF protein of this invention is substantially free of Factor VIIa activation activity, the induction of coagulation activity (i.e., as measured by clotting time) of plasma is proportional to the plasma concentration of VIIa. There is no "feedback" by the production of Factor VIIa from Factor VII present during the assay reactions.

Typically, the amount of time for a clot to form is proportional to the time of clotting for a preselected amount of Factor VIIa, and is determined by preparation of a standard curve, as is well known.

Thus, in one embodiment, the invention contemplates a method for detecting the presence, and preferably the amount, of Factor VIIa in a body fluid sample comprising the steps of:

a) admixing a preselected amount of said body fluid sample having Factor VIIa with a clotting assay admixture, wherein said clotting assay admixture

comprises a mutant human tissue factor (mhuTF) composition according to claim 5 and is substantially free of Factor VIIa and wild type human tissue factor, to form a Factor VIIa assay admixture;

b) maintaining said Factor VIIa assay admixture under conditions and a time period sufficient for said mhuTF to bind to any of said Factor VIIa in said sample and catalyze the formation of a clot; and

c) determining the amount of time required for the clot to form, which time is proportional to a predefined amount of Factor VIIa, thereby determining the presence, and preferably the amount, of VIIa present in said sample.

The components of a clotting assay admixture can vary widely so long as the addition of purified Factor VIIa, or VIIa present in the body fluid sample, to the admixture is sufficient to initiate a coagulation cascade and form a clot, typically according to conventional clotting time assays as is well known and also as described herein. A typical clotting assay admixture contains mhuTF and any buffered solution sufficient to support a coagulation cascade dependent upon Factor V, Factor IX, Factor X, Ca++, prothrombin, fibrinogen, and phospholipids as is well known. Because the patient's body fluid sample is typically plasma or blood that contains all of the other recited reagents necessary to support the coagulation cascade, (i.e., Factor V, Factor IX, Factor X, Ca++, prothrombin, fibrinogen, and phospholipids) a clotting assay admixture can be mhuTF and buffer alone where the sample is plasma or blood providing the other reagents necessary for Factor VIIa-dependent

coagulation.

A clotting assay admixture is substantially free of both wild type human tissue factor and Factor VIIa. By substantially free in the context of Factor VIIa is meant that background levels of clotting are observed when the clotting assay admixture is combined with a plasma sample that contains no detectable Factor VIIa. By substantially free in the context of wild type human tissue factor is meant that background levels of clotting are observed when the clotting assay admixture is combined with a plasma sample that contains Factor VII but no detectable Factor VIIa. A preferred clotting assay admixture is described in the Examples.

G. Diagnostic Kits

The present invention also describes a diagnostic system, preferably in kit form, for assaying for the presence and/or amount of one or more of the members of a huTF-mediated coagulation cascade in a sample according to the diagnostic methods described herein. A diagnostic system includes, in an amount sufficient to perform at least one assay, a subject mhuTF composition, as a separately packaged reagent.

Instructions for use of the packaged reagent are also typically included.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer

conditions and the like.

A diagnostic system of the present invention can also include a one or more of the other reagents used in the preparation of a clotting time assay as described herein, in an amount sufficient for at least one assay.

The reagent species of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packaging materials discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems, and can be formulated for single assay use, multiple assay use, manual or automated assay protocols, and the like.

The term "package" refers to a solid matrix or material such as glass, plastic (e.g., polyethylene, polypropylene and polycarbonate), paper, foil and the like capable of holding within fixed limits a diagnostic reagent such as a mhuTF composition of the present invention. Thus, for example, a package can be a bottle, vial, plastic and plastic-foil laminated envelope or the like container used to contain a contemplated diagnostic reagent.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of

the separate elements to be used in the method. For example, one of the container means may comprise a mhuTF composition of the invention. The kit may also have containers containing any of the other above-recited immunochemical reagents used to practice the diagnostic methods.

H. Therapeutic Methods

In view of the demonstrated ability of mhuTF to bind Factors VII or VIIa, and not activate Factor VII, mhuTF of this invention can be used therapeutically to prevent the activation of Factor VII. Inhibition of Factor VII activation is desirable where the reduction of Factor VIIA-dependent coagulation is indicated.

The method comprises contacting, in vivo or in vitro, Factor VII of Factor VIIa with an molar excess of mhuTF present in a therapeutic composition of this invention. In one embodiment, the contacting in vivo is accomplished by administering a therapeutically effective amount of a physiologically tolerable composition containing mhuTF of this invention to a patient, thereby contacting the Factor VII/VIIa present in the patient.

Thus, the present invention describes in one embodiment a method for inhibiting Factor VIIa-dependent coagulation in a human comprising administering to the human an immunotherapeutically effective amount of the mhuTF of this invention.

A representative patient for practicing the present methods is any human at risk for coagulation.

A therapeutically effective amount of a mhuTF is a predetermined amount calculated to achieve the

desired effect, i.e., to bind Factor VII/VIIa present in the patient, and thereby decrease the likelihood of coagulation in the patient. In the case of in vivo therapies, an effective amount can be measured by improvements in one or more symptoms associated with Factor VIIa-dependent coagulation.

Thus, the dosage ranges for the administration of a mhuTF of the invention are those large enough to produce the desired effect in which the symptoms of coagulated are ameliorated or the likelihood of coagulation are decreased. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art.

The dosage can be adjusted by the individual physician in the event of any complication.

A therapeutically effective amount of an mhuTF of this invention is typically an amount such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma or local concentration of from about 100 picomolar (pM) to 100 nanomolar (nM), preferably about 1 to 50 nM, and most preferably about 10 to 30 nM.

The mhuTF of the invention can be administered parenterally by injection or by gradual infusion over time. The mhuTF of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, dermally, and can be delivered by peristaltic means.

The therapeutic compositions containing a mhuTF

of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for in vivo therapies are contemplated.

Examples

The following examples relating to this invention

are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Construction of a Tissue Factor Expression Vector and Site-Directed Mutagenesis to Produce Human Tissue Factor Mutants (mhuTF)

The cell surface receptor tissue factor (TF) initiates coagulation by supporting the proteolytic activation of Factors X and IX as well as VII to active serine proteases. Architectural similarity of TF to the cytokine receptor family suggests a strand-loop-strand structure for TF amino acid residues 151-174 (SEQ ID NO 2). Site-directed mutagenesis as described herein of native human TF resulted in the production of the mutant human TF (mhuTF) proteins of this invention. Summarizing the results as described in Example 3, the Ala exchanges in the predicted surface loop demonstrated that residues Tyr^{157}, Lys^{159}, Ser^{163}, Gly^{164}, Lys^{165} and Lys^{166} are important for function (the amino acid residue positions of the amino acids are indicated). Addition of side chain atoms at the Ser^{162} position decreased function, whereas the Ala exchange was tolerated. The dysfunctional mutants bound VII with high affinity and fully supported the catalysis of small peptidyl substrates by the mutant TF-VIIa complex. Lys^{159} to Ala substitution was compatible with efficient activation of Factor X, whereas the Tyr^{157} to Ala

exchange and mutations in the carboxyl aspect of the predicted loop resulted in diminished activation of Factor X. The specific plasma procoagulant activity of all functionally deficient mutants increased 7- to 200-fold upon the supplementation of VIIa suggesting that TF residues 157-167 also provide important interactions that accelerate the activation of VII to VIIa. These data are consistent with assignment of the TF 157-167 region as contributing to protein substrate recognition and cleavage by the TF-VIIa complex.

A. Construction of the pCDM8 Expression Vector Containing the Human TF Coding Sequence

To construct an expression vector of this invention to provide for the subsequent mutagenesis and expression of tissue factor proteins, the cDNA containing the complete TF nucleotide coding sequence was ligated into the expression vector pCDM8 described by Seed, Nature, 329:840-842 (1987). The pCDM8 vector contained the following elements: a simian virus 40 (SV40) derived origin of replication; the eucaryotic transcription regulatory elements splice and poly(A)+; a bacterial episome origin of replication; and a procaryotic genetic marker (supF, suppressor tRNA gene); a polyoma origin of replication, bacteriophage M13 origin of replication, and the cytomegalovirus promoter (CMV).

The 775 base pair (bp) EcoR I fragment containing the cDNA (nucleotides 1 to 775) encoding amino acid residues 1-215 of TF was excised from the plasmid CTF545 prepared as described by Morissey et al., Cell, 50:129-135 (1987), the disclosure of which is hereby

incorporated by reference. This resulting fragment was ligated into the 505 bp EcoR I-Hind III fragment of pCTF439 consisting of nucleotides 776 to 1280 that encode the amino acid residues of TF from 216-263 and the concatenated DNA was then cloned into pUC19 to yield the construct pCTF553 as described by Rehemtulla et al., Thrombosis and Haemostasis, 65:521-527, (1991), the disclosure of which is hereby incorporated by reference. The resulting fragment was then cloned into pUC18 using BamH I linkers to form pCTF1200. The sequence of this construct confirmed the presence of the entire coding region of native human TF in addition to 360 bp of untranslated 3' region and 38 bp of untranslated 5' sequence. For expression in Chinese Hamster Ovary cells (CHO), the BamH I insert from pCTF1200 was excised, blunt-ended using Klenow fragment of DNA polymerase and ligated into the vector pCDM8 prepared as described above that had been digested with Xho I and blunt-ended with Klenow. The resultant construct, designated pETF1773, contained the TF cDNA in an orientation that allowed transcription under the control of the strong CMV promoter of pCDM8. The complete nucleotide sequence of the pCDM8 vector containing the TF cDNA insert is listed in SEQ ID NO 1. The nucleotide sequence encoding the signal peptide of TF begins at nucleotide position 2267 and ends at 2362 followed by the nucleotide sequence encoding TF beginning at 2363 and ending at 3154. The encoded 263 amino acid residue sequence of the nonmutagenized native human TF is listed in SEQ ID NO 2. Other expression vectors having equivalent elements are contemplated for use in this invention.

B. Mutagenesis of TF

Oligonucleotide-directed mutagenesis was performed using the uracil substitution method according to Kunkel, Proc. Natl. Acad. Sci., USA, 82:488-492 (1985) and also described in Ausebel et al., Current Protocols in Molecular Biology, Unit 8, Wiley and Sons, New York, (1990). The following modifications to the basic site-directed mutagenesis procedure were performed to adapt the procedure for use with the pCDM8 expression vector as described by Rehemtulla et al., J. Biol. Chem., 266:10294-10299 (1991). Phosphorylated mutagenic oligonucleotides (10 nanograms (ng)) were separately annealed to single-stranded template (100 ng) which was isolated from the strain CJ236/p3 (Invitrogen, San Diego, CA) in 20 mM Tris-HCl at pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl at 70 degrees Celsius (70C) and cooled to room temperature. The second strand was synthesized using T4 DNA polymerase and T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM deoxynucleotide triphosphates and 10 mM ATP for 5 minutes at 4C, 5 minutes at room temperature, and 90 minutes at 37C. One-fifth of the reaction was then transformed into MC1061/p3 and transformants were screened for the presence of mutant sequences by restriction enzyme analysis to detect newly introduced restriction sites, if applicable. Sequencing of mutant constructs was accomplished using single-stranded DNA rescued from the strain XS127 and the helper phage R408. The Sequenase system (US Biochemical, Cleveland, OH) was used to perform dideoxynucleotide sequencing.

The following oligonucleotides listed in the 5' to 3' direction were used in the above mutagenesis procedure to obtain the corresponding identified TF mutants. The triplets for introducing the desired amino acid substitutions at the locations indicated in the TF mutant are underlined. Mutant $TF_{A157A159}$ was generated with the oligonucleotide TATACACTTTACGCGTGGGCATCTTCAAGT (SEQ ID NO 3); $TF_{A161D162A163}$ with TTGGAAATCTGCAGATGCAGGAAAGAAA (SEQ ID NO 4); TF_{A161} with GGAAATCTGCCTCGAGTGGAAAGAA (SEQ ID NO 5); TF_{A162} with AATCTTCAGCCTCGGGAAAGAA (SEQ ID NO 6); TF_{A163} with TGGAAATCCTCGAGTGCAGGAAA (SEQ ID NO 7); TF_{A164} with TATTGGAAATCCTCGAGTTCAGCAAAGAAAACA (SEQ ID NO 8); TF_{A167} with TCTTCAAGCTCAGGAAAGAAAGCAGCCAAA (SEQ ID NO 9); TF_{T162} with TGGAAATCTTCAACCTCGGGAAAGAAA (SEQ ID NO 10); TF_{D162} with TGGAAATCTTCAGACTCGGGAAAGAAA (SEQ ID NO 11); TF_{N162} with TGGAAATCTTCAAACTCGGGAAAGAAA (SEQ ID NO 12); $TF_{A151A153A155}$ with TGGCAAGGACGCGATCGCTACAGCTTATTATTGG (SEQ ID NO 13); $TF_{A169A171A173}$ with AAAACAGCTGCAACAGCCACTGCTGAGTTT (SEQ ID NO 14); $TF_{A170A172A174}$ with GCCAAAGCAAACGCTAATGCGTTTTTGATCGATGTG (SEQ ID NO 15); $TF_{R158G160}$ with CTTTATTATCGAAAAGGTTCAAGTTCA (SEQ ID NO 16); TF_{A159} with TATTATTGGGCATCCTCGAGTTCAGGA (SEQ ID NO 17); $TF_{R158A159G160}$ with CTTTATTATCGCGCAGGTTCAAGTTC (SEQ ID NO 18); $TF_{G152I154T156}$ with GGCAAGGACCTCGGGTATATACTTACTTATTGGAAA (SEQ ID NO 19); $TF_{G152I154T156R158G160}$ with GGCAAGGACCTCGGGTATATACTTACTTATCGGAAA (SEQ ID NO 20) used in combination with the $TF_{R158G160}$ oligonucleotide in SEQ ID NO 17; TF_{A157} with TATACACTTTACGCGTGGAAATCT (SEQ ID NO 21); $TF_{A149A150}$ with TGTTTTTGGCGCAGCTTTAATTTATA (SEQ ID NO 22); $TF_{A161D162A163}$

with TTGGAAATCTGCAGATGCAGGAAAGAAA (SEQ ID NO 23); $TF_{A165A166}$ with AAGTTCAGGCGCGGCAACAGCCAAAA (SEQ ID NO 24); TF_{A147} with CGGGATGTAGCTGGCAAGG (SEQ ID NO 25); and $TF_{A176A178A180}$ with ATGAGTTTGCGATCGCTGTGGCTAAAGGAG (SEQ ID NO 26). Mutations were confirmed by DNA sequencing of CsCl purified plasmid DNA which was subsequently used for the transfection experiments.

C. Production of Stable Cell Lines

For the production of stable cell lines, Chinese Hamster Ovary cells (CHO-K1) having an ATCC Accession No CCL61 were grown in Dulbecco's modified Eagles medium (DMEM), 10% newborn calf serum (HyClone Laboratories, Logan UT), 2 mM l-glutamine, 0.1 mM proline, 100 U/ml penicillin and 100 ug/ml streptomycin. The prepared cells were transfected using the calcium phosphate precipitation method and stable cell lines were generated by separately cotransfecting 1 ug of a neomycin resistance gene for selection purposes (pMAMneo, Clontech Laboratories, San Francisco, CA) with the 20 ug each of the purified mutagenized plasmids prepared in Example 1B encoding the mutations described in Example 1B. The DNA was first diluted in 250 mM $CaCl_2$ then precipitated by drop-wise addition of 2X Hepes buffered saline (0.25 M NaCl, 40 mM Hepes, 0.7 mM Na_2HPO_4 at pH 7.05 with 0.5 M NaOH) followed by vigorous vortexing and then incubation at room temperature for 20 minutes. The precipitate was added to a monolayer to CHO-K1 cells. After maintenance at 37C for 4 hours, the cells were treated with 2 ml of 10% glycerol in medium for 3 minutes and then washed three times with PBS (10 mM sodium phosphate at pH 7.4 and 0.15 M NaCl). The

washed cells were then maintained with fresh medium for 36 hours in transient transfection experiments.

To select for transfectants and maintain stable cell lines, 36 hours after transfection, the cultures were suspended and diluted to a density of 10^4 cells/100 mm petri dish. The medium was supplemented with 600 ug/ml G418 (Geneticin, Gibco, Gaithersburg, MD) and the cells were replated in petri dishes. Fresh medium was added to the cells after 7 days and G418 resistant colonies appeared after 10-14 days. Single colonies were picked using cloning cylinders and grown in large cultures for analysis. Tunicamycin (Sigma, St. Louis, MO) treatment of the cells was performed at 1 ug/ml for 48 hours.

2. Expression of Tissue Factor Mutants from the Mutagenized Expression Vectors

The stable cell lines expressing mutant TF prepared in Example 1C were then grown to allow for purification of the recombinant mutant TF. To accomplish this, the stable cells lines were separately grown in 2 liter spinner flasks in Excell 301 (JR Scientific, Woodland, CA), 10% newborn calf serum (Hanna Biologicals), 2 mM L-glutamine, 0.1 mM proline, 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were harvested at maximum density and lysed in 200 ml of 1% Triton X-100 in TBS. After complete lysis, insoluble debris was pelleted at 10,000 X g at 4C for 20 minutes. The resultant supernatant was applied to an immunoaffinity column as described by Morissey et al., Cell, 50:129-135 (1987), the disclosure of which is hereby incorporated by reference. The unbound material was washed from the

column using TBS, 0.1% Triton X-100 and followed by 0.1 M glycine at pH 4.5, containing 0.1% Triton X-100.

The TF mutant proteins were separately eluted with 0.1 M glycine at pH 2.5 containing 0.1% Triton X-100. Fractions containing the eluted mutant TF proteins were immediately neutralized to pH greater than 5.5 and rapidly dialyzed against 0.01% Triton X-100 in TBS for storage at -70C. Concentration of the eluted proteins was determined by immunoassay and by direct protein using the BCA protein assay (Pierce, Rockford, IL). All assays were standardized with purified natural human TF quantitated by amino acid composition based on a protein mass of 29,593 as described by Morissey et al., supra.

Expression of mutant TF ranged from 0 to 970 ng per 10^6 cells equivalent to that seen with normal TF as described by Rehemtulla et al., Thrombosis and Haemostasis, 65:521-527 (1991).

3. Determination of a Functional Tissue Factor Mutant

For the assays described herein, the coagulation proteins were purified as described Ruf et al., J. Biol. Chem., 266:2158-2166 (1991). VIIa was purchased from Novo Nordisk (Gentofte, Denmark) and the functional activity and binding characteristics of the recombinant protein have previously been described by Ruf et al., J. Biol. Chem., 266:15719-15725 (1991). Coagulation factor deficient plasmas were purchased from George King Bio-medical. The chromogenic substrate, Spectrozyme FXa, was from American Diagnostica Inc. (Greenwich, CT).

A. Assay for Specific Functional Activity of TF Mutants

Specific functional activity was determined for transiently expressed mutants prepared as described in Example 1. First, TF antigen in a detergent (CHAPS) cell lysate was determined by immunoassay using two non-overlapping monoclonal antibodies or, alternatively, polyvalent antibody purified by affinity for immobilized TF as capture antibody followed by detection with monoclonal antibody. The purification and assays were performed as described by Ruf et al., J. Biol. Chem., 266:2158-2166 (1991), the disclosure of which is hereby incorporated by reference. The assay was calibrated with recombinant human TF prepared as described for mutant TF in Example 1. Initiation of coagulation by wild-type and mutant TF in recalcified plasma was determined after lysis of cell pellets from 2 X 10^6 cells/ml with 15 mM octyl-glucopyranoside in HBS for15 minutes at 37C followed by 3-fold dilution according to Rehemtulla et al., Biochem. J., 282:737-740 (1992). Briefly, clotting times were determined for the cell lysates in a one stage clotting assay containing equal volumes of sample, plasma, lysate and 20 mM $CaCl_2$ and converted to units based on a calibration curve established with purified TF reconstituted in phospholipid vesicles (70% phosphatidylcholine, 30% phosphatidylserine) using detergent solubilization and dialysis, as described in detail by Ruf et al., Thrombosis and Heamostasis, 66:529-533 (1991). Both the ELISA and clotting assays were quality controlled using cell pellets of a stable cell line expressing wild-type TF prepared as

described for stable cell lines expressing mutant TF proteins of this invention. Samples of this cell line were subjected to the same lysis and analysis procedure as the test samples. The coefficient of variation calculated for a one month sampling period was 10.1% (n = 6) for the ELISA and 10.8% (n = 16) for the clotting assay.

The results of the assays are presented in Table 1. The specific functional activity was calculated based on the determinations of the TF antigen by ELISA and for functional activity in the one stage clotting assay. The mean and standard deviation were calculated for the indicated number (n) of duplicate determinations is given. The specific functional activity relative to wild-type TF is given as % of wild-type.

Table 1

TF mutant	% of wild-type	specific activity (mU/ng)	n
wild-type	100	298 ± 87	6
$TF_{A151A153A155}$	3	10 ± 8	3
$TF_{A169A171A173}$	66	197 ± 99	5
$TF_{A170A172A174}$	111	332 ± 130	7
$TF_{A157A159}$	2	5 ± 8	4
TF_{A161}	128	383 ± 168	4
TF_{A162}	82	244 ± 107	4
TF_{A163}	11	33 ± 9	4
TF_{A164}	3	8 ± 7	4
TF_{A167}	95	282 ± 89	3
TF_{T162}	19	58 ± 15	4
TF_{N162}	21	64 ± 51	4

TF_{D162}	3	8 ± 5	3
$TF_{A161D162A163}$	2	7 ± 2	4

Tyr^{157} and Lys^{159} have previously been identified as functionally important and are flanked in linear sequence by the non-critical residues Tyr^{156} and Ser^{160} as described by Rehemtulla et al., Biochem. J., 282:737-740 (1992). Ala replacements for Tyr^{157} or Lys^{159} resulted in a 87% or 92% respective loss of specific functional activity by plasma coagulation assay. Replacement of both residues in one mutant reduced the functional activity by 98% as shown in Table 1 which may indicate an additive effect of the two mutations. Ala replacements for Ser^{161} and Ser^{162} did not result in significant loss of function. In contrast, Ala substitution for Ser^{163} reduced specific functional activity by 89%, indicating importance of the Ser^{163} side chain.

A recent study indicated that the positively charged residues Lys^{165} and Lys^{166} are important for recognition and activation of the protein substrate X. See, Ruf et al, J. Biol. Chem., 267:6375-6381 (1992). The charge requirements were further explored in the vicinity of these two Lys residues by introducing Asp, thus a negative charge in place of Ser^{162}. Although Ser^{162} could be replaced by Ala without loss of function, the TF_{D162} mutant demonstrated a significant loss of function (Table 1). A similar loss of function was also observed with the triple mutant $TF_{A161D162A163}$. Within the experimental error of this analysis, the additional Ser^{163} to Ala substitution thus appeared to be silent in this latter mutant. Other replacements for Ser^{162} further demonstrated that substitution by bulkier side chains, as in Thr or Asn, resulted in nearly 80% loss of function. This suggests that the packing of the Ser^{162} side

chain may be critical for local conformation. Alternatively, Ser^{162} could be localized within an interactive surface without direct functional contribution and the additional side chain atoms may not be accommodated upon assembly of the interacting protein surfaces. Since there was an additional effect when a negative charge was introduced at the Ser^{162} position, this may indicate that the local charge field contribution of the adjacent Lys^{165} and Lys^{166} was perturbed or the side chain orientation of these residues was distorted. These two Lys residues are separated in linear sequence from the stretch of Ser residues by Gly^{164}.

Gly^{164} appears to be necessary for function of the 157-167 region, since the Gly^{164} to Ala substitution resulted in very low specific functional activity (Table 1). Gly residues are often found in reverse turns as described by Creighton et al., Proteins, W.H. Freemann and Company, New York (1984), because of the lack of a C--atom, the increased flexibility of their backbone and their more favorable phi and psi angles. The functional defect resulting from the Gly^{164} to Ala exchange is likely to reflect local perturbation of the orientation of adjacent functionally important residues. Thr^{167} could be replaced by Ala without alterations in the functional properties of TF. In addition, the TF mutant $R^{158}G^{160}$ exhibited overall specific functional activity of 41 +/- 15.

This analysis therefore provides evidence for several functionally important residues in the 157-167 region of TF as shown in Figure 1 where the schematic representation of residues 151-172 in TF is illustrated. Alignment to strand C according to Bazan, Proc. Natl. Acad. Sci., USA, 87:6934-6938 (1990) is indicated and the functionally important residues are highlighted. The single letter code

for amino acids is used. Analysis of selected mutants stably expressed on CHO-cells was then performed as described below to further define functional defects.

B. Assay for Factor VII Binding of TF Mutants

Dysfunction of a TF mutant could follow from reduced affinity for its ligand VII/VIIa. To examine this possibility, the binding of VII to the various mutant proteins in radioligand binding analyses on cell monolayers. The binding characteristics of VII and VIIa to cell surface expressed mutant or wild type TF were determined essentially as described by Fair et al., J. Biol. Chem., 262:11692-11699 (1987) and further described by Rehemtulla et al., J. Biol. Chem., 266:10294-10299 (1991). Briefly, stable cell lines produced in Example 1 expressing mutant TF as well as a control normal TF were seeded at equal densities into 24-well tissue culture dishes (Costar). At confluence, the cell monolayers were washed three times and maintained with plasma derived ^{125}I-VII/VIIa, in the presence of 5 mM $CaCl_2$, 0.5% BSA in 10 mM Hepes, 150 mM NaCl, 4 mM KCl, 11 mM glucose, pH 7.4. Nonspecific binding was determined in the presence of 50-fold molar excess of a monoclonal antibody against TF (TF9-6B4) which completely blocks the binding of VII to TF. Bound radioactivity was determined after rapidly washing the cells and solubilizing the monolayer. Duplicate determinations from at least three experiments were used for Scatchard analysis which was performed using the LIGAND program, as described by Rehemtulla et al., J. Biol. Chem., 266:10294-10299 (1991).

The results of the binding assays are shown in Table 2 and Figure 2. The dissociation constant (K_d) and maximal number of sites +/- the error estimate determined by the LIGAND program are given and were calculated from n

independent experiments.

Table 2

Mutant	K_d (nM)	binding sites (fmol/well)	n
wild-type	10.2 ± 1.4	800 ± 200	4
TF_{A157}	5.8 ± 2.0	500 ± 200	5
TF_{A159}	8.9 ± 1.7	1700 ± 200	3
$TF_{A161D162A163}$	6.8 ± 0.7	900 ± 100	8

Previously, characterization of the $TF_{A165A166}$ mutant had demonstrated normal binding of VII, based on radioligand binding to cell surface TF and to detergent solubilized TF in the absence of phospholipid, as well as based on dissociation analysis of the TF-VIIa complex. See, Ruf et al, J. Biol. Chem., 267:6375-6381 (1992). These observations indicated that the basic residues Lys^{165} and Lys^{166} do not contribute significantly to the binding energy required for assembly of the TF-VIIa complex. Since removal of charged side chains, as in the $TF_{A165A166}$ mutant, may be tolerated more readily than the addition of an oppositely charged or bulkier side chain in the same region, the VII binding characteristics of $TF_{A161D162A163}$. This mutant was chosen because it exhibited the greatest loss of function, when mutants at the Ser^{162} and Ser^{163} position were compared

Radioligand binding analysis with VII/VIIa resulted in similar binding profiles for wild-type TF and $TF_{A161D162A163}$ as shown in Figure 2 specific binding of VII to cell surface $TF_{A161D162A163}$ (A) and wild-type TF (B) is shown. The insets give the Scatchard analysis for the same data obtained in a

representative experiment. The dissociation constant derived from Scatchard analysis demonstrated high affinity binding of VII by the mutant TF (Table 2). These data suggest that the significant loss of specific function of $TF_{A161D162A163}$ cannot be attributed simply to decreased affinity for VII. The binding analysis for $TF_{A161D162A163}$ may be taken as representative for the TF_{A163} mutant and mutants with other substitutions for Ser^{162} which exhibited less profound decreases in specific functional activity. This provides additional evidence that the carboxyl aspect of the putative 157-167 loop in TF is not required for binding of VII.

The binding of VII to the TF_{A157} and TF_{A159} mutants was further analyzed. These two mutants lack the side chains of the functionally important residues Tyr^{157} and Lys^{159}. Both mutants bound VII with undiminished affinity compared to wild-type TF which was analyzed in parallel (Table 2). Thus, from these data, one can conclude that the functionally important residues in the amino aspect of the putative 157-167 surface loop are not required for high affinity VII binding.

C. Assay of Catalytic Function by TF Mutants

To further characterize the mutant TF proteins of this invention, cleavage of small peptidyl substrates by the TF-VIIa complex was analyzed using lysates of cell lines stably expressing wild-type or mutant TF. Cells were lysed with 4 mM CHAPS dissolved in TBS and this lysate was diluted two-fold in the final reaction (200 ul) which contained VIIa (10 nM), $CaCl_2$ (5 mM) and Spectrozyme FXa (1.25 mM). The rate of Spectrozyme hydrolysis was determined at ambient temperature in a kinetic plate reader (Molecular Devices, Mountain View, CA).

The ability of the dysfunctional mutants to induce

catalytic (amidolytic) function of VIIa when assembled with TF was analyzed with detergent lysates of cells expressing the mutant TFs. When VIIa in excess was incubated with identical concentrations of wild-type or mutant TF, comparable hydrolysis of the chromogenic substrate Spectrozyme FXa was observed as shown in Figure 3 which shows the amidolytic and proteolytic activity of mutant TF-VIIa complexes. In Figure 3A, the cleavage of small peptidyl substrates was assessed with Spectrozyme FXa in the presence of 10 nM VIIa and 5 nM wild-type or mutant TF. The rate of hydrolysis of the peptidyl substrate was determined in a 200 ul reaction with a kinetic plate reader and is given as the increase in absorbance (mOD/min). In addition to the data presented in Figure 3 for the four mutants shown, (TF_{A157}, TF_{A159}, $TF_{A161D162A113}$ and $TF_{A165A166}$), the mutant $TFR_{158G160}$ exhibited similar activity having a mean amidolytic activity of 7 +/- 1.3. The data further supports the binding analysis that all TF mutants in the 157-167 region form equivalent complexes with VIIa. This analysis also excludes the notion that a significant fraction of the mutant TF is misfolded and non-interacting with VIIa. In addition, these data demonstrate that the catalytic function of VIIa towards small peptidyl substrates is normal indicating a fully functional catalytic triad in VIIa when complexed with the mutant TFs.

D. Proteolytic Activity of Mutant TF-VIIa Complexes

The mutant TF proteins of this invention were further analyzed by their ability to activate Factor X which is a property mediated by normal TF. For the assay, X activation was analyzed by incubating a freshly prepared octyl-glucopyranoside cell lysate (0.03 to 0.12 nM TF) with excess VIIa (5 nM) at 5 mM $CaCl_2$ for 5 minutes at 37C

followed by addition of Factor X (1 uM). Samples were removed from the reaction and quenched in 100 mM EDTA in TBS (20 mM Tris-HCl, 140 mM NaCl, pH 7.4). Xa in the quenched reaction was determined with Spectrozyme FXa and the rate of Xa generation was calculated for several points in the initial linear portion of the progress curve as described by Ruf et al., J. Biol. Chem., 266:2158-2166 (1991).

The results of the assay are shown in Figure 3B where the activation of X (1 uM) by mutant and wild-type TF in the presence of excess VIIa (5 nM) was determined at 37C. The rate of Xa formation per enzymatic unit TF-VIIa was calculated based on the TF concentration in the assay. The mean and standard deviation calculated for three independent experiments are shown in both panels. For all the stable cell lines described in this report, the activation of X on viable cell surfaces was comparable to the experiments with octyl-glucopyranoside lysed cells. This indicated that no loss of functional activity was introduced by the detergent lysis procedure.

At identical TF concentrations, TF_{A159}-VIIa and wild-type TF-VIIa complexes activated X at rates that were indistinguishable. In contrast, complexes formed with $TF_{A161D162A163}$, TF_{A157} or $TF_{A165A166}$ demonstrated rates of X activation which were reduced by 85%, 55%, and 59%, respectively as shown in Figure 3B. Thus, all dysfunctional mutants with the exception of TF_{A159} formed catalytic complexes with VIIa that exhibited some loss of proteolytic activation of the natural protein substrate. Amidolytic activity of the mutant TF-VIIa complexes was indistinguishable from that of the wild-type TF-VIIa complex suggesting that the mutants have a selective defect either in extended recognition and hydrolysis of protein substrates, or in the release of Xa, the cleaved product.

The $TF_{R158G160}$ mutant exhibited similar proteolytic characteristics having a value of 1.33 +/- 0.27.

To evaluate the role of the alternative substrate for TF-VIIa, namely IX, during the activation of the extrinsic coagulation pathway by the TF mutants, the specific functional procoagulant activity in normal and IX deficient plasma was compared. Both, wild-type TF and the TF_{A159} mutant demonstrated a similar and less than two-fold decrease of specific procoagulant activity in IX deficient plasma compared to normal plasma as shown in Figure 4. The specific functional activity was determined in normal (Figure 4A) or IX deficient plasma (Figure 4B) with (hatched bars) or without (filled bars) 500 nM VIIa added. The mean and standard deviation were calculated from three experiments.

The diminished function in IX deficient plasma was more pronounced with the TF_{A157}, $TF_{A161D162A163}$, and $TF_{A165A166}$ mutants resulting in a 5-, 2.5- and 3.1-fold reduction of specific functional activity as shown in Figure 4. Thus, it appears that mutants with a defect in X activation are even less active in the absence of IX suggesting modest compensation of the functional defect by IX. This is consistent with the proposal that the generation of IXalpha by Xa and the accelerated activation of IXalpha to IXaalpha by TF-VIIa plays a role in the initiation of the coagulation cascade by TF as described by Lawson et al., J. Biol. Chem., 266:11317-11327 (1991).

E. Assay to Measure the Conversion of Factor VII to Factor VIIa

The inability of the mutant TF proteins of this invention to convert the substrate VII to VIIa is the critical factor for the function of the proteins in a

standard hospital clotting assay where it is essential to only measure the amount of VIIa currently present in the plasma and not the amount of VIIa converted from VII as the clotting assay progresses. Clotting activity of TF mutants with or without added VIIa was evaluated with freshly prepared octyl-glucopyranoside lysates. Cell lysate (100 ul), normal or coagulation factor deficient plasma (50 ul) and 500 nM VIIa or buffer (50 ul) were equilibrated at 37C for 1 minute followed by initiation of the reaction by adding 20 mM $CaCl_2$ (100 ul). The VIIa concentration was chosen to provide a 50 to 100-fold excess over VII in the plasma. Control experiments with a 10-fold lower concentration of VIIa gave similar results. Further, preincubation of wild-type and mutant TF with VIIa in the presence of $CaCl_2$ followed by the addition of plasma did not reveal differences compared to the assay where the reaction was started by the addition of Ca^{2+}. This suggests that a slower assembly of VIIa with the TF mutants does not contribute to the functional defect. Functional activity was derived from double logarithmic calibration curves of serial dilutions of purified and phospholipid reconstituted TF versus the clotting times in normal or factor IX (IX) deficient plasma. The concentration of TF which produced a 50 seconds (s) clotting time in normal or IX deficient plasma was set to 1U/ml of TF activity. Specific functional activity was based on the TF antigen concentration of the cells determined by ELISA. Several dilutions of mutant TF (30 to 200 pM) were used to establish the functional activities for each experimental condition, and mean and standard deviation were calculated for three independent experiments.

The potential contribution of TF residues 157-167 to the conversion of VII to VIIa was analyzed in the above

plasma coagulation assay. The specific functional activity of the TF mutants relative to wild-type TF was determined in normal and IX deficient plasma with or without a 50- to 100-fold molar excess of recombinant VIIa relative to VII present in the plasma. With wild-type TF, addition of VIIa accelerated the clotting times in both normal and IX deficient plasma. Based on calibration curves with purified TF in normal or IX deficient plasma, specific functional activity increased 3-fold in normal human plasma and 5.4-fold in IX deficient plasma (Figure 4). The specific activity of all TF mutants increased to a much greater extent in the presence of VIIa suggesting a contribution of residues in the 157-167 surface loop to the activation of VII during the TF dependent initiation of coagulation. In the presence of VIIa, the functional activity of $TF_{A161D162A163}$, $TF_{A165A166}$, or TF_{A157} increased 200-, 198- or 7-fold in normal plasma, and 566-, 653- or 37-fold in IX deficient plasma. The specific functional activity of these mutants was not completely normalized by the addition of VIIa, but remained reduced 40- to 45-fold for $TF_{A161D162A163}$, 14-fold for $TF_{A165A166}$, and 5-fold for TF_{A157} in comparison to wild-type TF (Figure 4). The decreased activity in the presence of VIIa is consistent with the diminished rate of proteolytic activation of X demonstrated for all three mutants. Since addition of VIIa increased the specific functional activity of all mutants defective in X activation, it is suggested that the feed-back loop of Xa cleaving VII bound to TF may be of importance.

The same residues of TF which are important for TF cofactor function in the activation of X may also contribute to recognition of VII as a substrate by the TF-VIIa complex. Evidence for a specific contribution of cofactor residues to this latter activation is provided by the mutant TF_{A159} which

fully supported activation of X. In both, normal and IX deficient plasma, VIIa increased the specific functional activity of TF_{A159} 11- or 26-fold which is a 4- to 5-fold greater increase than observed with wild-type TF. The activity of TF_{A159} in the presence of high concentrations of VIIa was similar to wild-type TF without added VIIa, and a 2.8-fold difference remained when mutant and wild-type TF activity were compared in the presence of excess VIIa. Despite this unexplained difference, the normalization of the functional defect of TF_{A159} by the addition of VIIa suggests that Lys^{159} is important for the conversion of VII to VIIa. Conceivably, this residue may be critical for assembly of Xa with the TF-VII complex during activation of the bound VII, or Lys^{159} may be important for recognition and hydrolysis of the substrate VII by the TF-VIIa complex. The TF mutants described here may aid in elucidating the specific contribution of cofactor residues to the auto-activation of VII by VIIa.

The dependency of the mutant TF proteins of this invention on VIIa for the formation of a clot in the clotting assay was then determined to provide the definitive analysis of those mutant proteins that bind to both VII and VIIa, have amidolytic and proteolytic activity but do not activate the conversion of VII to VIIa. Thus, if the mutant TF proteins exhibited a significant enhancement of clotting time in the presence of added VIIa over the clotting times in its absence, then the mutant TFs did not activate the conversion of VII to VIIa despite binding to the VII substrate. Those that exhibit the enhanced clotting only in the presence of added VIIa are said to exhibit VIIa dependency.

For this assay, cells expressing mutant TF proteins prepared in Example 1 were lysed with standard detergent

lysis protocol as described in Ruf et al., J. Biol. Chem., 267:6375-6381 (1992), the disclosure of which is incorporated by reference, and familiar to one of ordinary skill in the art. The reaction admixture contained 50 ul human plasma, 50 ul added recombinant VIIa or buffer control, and 100 ul cell lysate containing the mutant TF proteins. The reaction was started by the admixture of 100 ul 20 mM $CaCl_2$. The clotting time was then determined and the shortening of the clotting time caused by mutant TF in the presence or absence of VIIa was measured in comparison to that seen with wild type TF. The results of the assay are shown in Table 3.

Table 3

Mutant TF*	Clotting Time no VIIa	Clotting Time 10 nM VIIa	Dependency on Added VIIa
R158,G160	145 s	60 s	yes
A159	49 s	37 s	yes
R158,A159,G160	365s	240s	yes
G152YILT	125 s	105 s	no
G152YILTYRKG	145 s	90 s	yes
A157	98 s	61 s	yes
A149,A150	173 s	156 s	no
A161,D162,A163	400 s	150 s	yes
A165,A166	295 s	199 s	yes
A147	230 s	152 s	yes
A176,A178,A180	125 s	105 s	no

* The above clotting times and dependency on added

VIIa is compared to results with wild type TF where without VIIa the clotting time was 45 s and with 10 nM VIIa the clotting time was 41 s. Thus, wild type is not dependent on added VIIa.

VIIa dependency is maintained in the framework of other mutations. The G152YILT mutant ($TF_{G152I154T156}$) is not sensitive for VIIa but the addition of the R158KG160 mutation ($TF_{R158G160}$) renders the mutant TF VIIa sensitive and results in a mutant with the desired function lacking the ability to convert VII to VIIa while having the functions of amidolytic and proteolytic activity as described herein. The mutants that are VIIa dependent and exhibit overall clotting activity as measured by a decrease in clotting time as shown in Table 3 thus preferred for use in a rapid and efficient screening assay such as the one described in Example 3F. The most preferred mutant TF proteins of this invention contained the mutation at amino acid position 158 where a tryptophan has been changed to an arginine and at amino acid position 160 where a serine has been changed to a glycine.

F. Prothrombin Time Assay

The prothrombin time assay measures coagulation factors of the extrinsic pathway. These include Factors VII, X, V, II and I. Some of these factors are affected by oral anticoagulant drugs. The prothrombin time assay was thus useful for monitoring oral anticoagulant therapy. For this assay utilizing the mutant TF proteins of this invention, an operator of a typical coagulation instrument distributed by commercial entities such as Ortho Diagnostics only has

to pipette 0.1 ml of patient plasma sample into an assay cuvette well and place the cuvette into the instrument. As the automatic carousel indexes through the incubation plate, the samples are warmed to 37.5C. The reagent arm then dispenses 0.2 ml prewarmed mutant TF reagent of this invention and clotting times are measured. The remainder of the assay is automated as is the data reduction. The replacement of normal TF (thromboplastin) with the mutant TF proteins of this invention along with corresponding instrument software changes would support data reduction to yield the accurate concentration of Factor VIIa present in the blood without any confounding by the amount of Factor VIIa resulting from the conversion of VII effected by normal TF.

4. Analysis of Tissue Factor Mutants

A. Mutational Support for the Predicted Strand-Loop-Strand Structure in TF Residues 151-174

Structural prediction suggested that residues 157 through 167 are localized in a surface loop of the TF carboxyl module between the equivalents of the C and D beta-strands in the immunoglobulin nomenclature as described by Bazan et al., Proc. Natl. Acad. Sci., USA, 87:6934-6938 (1990). Based on this structural model, residues 151-156 should form a beta-strand and residues Leu^{151}, Tyr^{153} and Leu^{155} in TF are alternating hydrophobic residues consistent with the model. Residues Ile^{152}, Thr^{154} and Tyr^{156} may form the hydrophilic side of the beta-strand and hydrophilic substitutions for Ile^{152} and Tyr^{156} are found in the TF sequence of other species as described

by Andrews et al., Gene, 98:265-269 (1991). The hydrophobic residues Leu^{151}, Tyr^{153} and Leu^{155} were each replaced by Ala and the triple mutant TF was transiently expressed. In several experiments described in Example 3, $TF_{A151A153A155}$ was expressed at levels one tenth or less of a wild-type TF control transfected in parallel. This suggested diminished efficiency of cellular processing which may indicate alteration of the protein fold. See, Bass et al., Proc. Natl. Acad. Sci., USA, 88:4498-4502 (1991) and Pakula et al., Proc. Natl. Acad. Sci., USA, 83:8829-8833 (1986). Further, Western-blot analysis of the mutant protein exhibited increased electrophoretic mobility characteristic of incomplete glycosylation of TF. These data are consistent with a structurally altered mutant protein which undergoes less than normal cellular processing. The specific functional activity of $TF_{A151A153A155}$ was greatly reduced (Table 1). Since three alternating hydrophobic residues in a predicted beta-strand had been replaced, one or more side chains which are critical for the hydrophobic core of the carboxyl module may have been removed. This mutational analysis is consistent with the predicted beta-strand architecture of the TF extracellular domain. Mutational Cys to Ser exchange resulted in a lack of covalent stabilization of the disulfide bonded loop (residues 186-209) resulted in a protein with quantitatively reduced cell surface expression and diminished specific functional activity as described by Rehemtulla et al., J. Biol. Chem., 266:10294-10299 (1991). These two examples of an apparent structural defect coupled with loss of functional activity demonstrate an important

functional role of the carboxyl module of TF.

The sequence 168-174 in TF which corresponds to the predicted D beta-strand is hydrophilic. The D-strand in immunoglobulins and structurally related proteins described by Williams et al., Ann. Rev. Immunol., 6:381-405 (1988) is located at the edge of the opposed two beta-sheets and often lacks residues which interact with the hydrophobic core. In the growth hormone structure the corresponding residues were found to form a short strand which aligned with the opposite bata-sheet as described by De Vos et al., Science, 255:306-312 (1992). Two mutants were generated which replaced alternating residues. In the first mutant Thr^{170}, Thr^{172} and Glu^{174} were replaced by Ala, the second mutant had replacements of Lys^{169}, Asn^{171} and Asn^{173} oriented to the opposite site of the predicted beta-strand. Both triple mutants exhibited quantitatively normal cellular expression, indicating little if any structural alterations. In addition, residues 168-174 were not required for function of TF, as indicated by normal specific functional activity (Table 1). Although the effect of mutational exchange of residues 169-174 can be considered as consistent with the proposed structural model, elucidation of the three dimensional structure of the TF extracellular domain will be required to establish the structural alignment of residues in this region of TF.

B. Implications of TF Structure Relating to Function

The region of TF which includes residues 151-174 has been predicted to adopt a strand-loop-strand structure, from sequence based

secondary structure predictive algorithms as described by Bazan et al., Proc. Natl. Acad. Sci., USA, 87:6934-6938 (1990) and apparent homology to the growth hormone receptor structure as described by De Vos et al., Science, 255:306-312 (1992). The previously demonstrated predominant beta-strand secondary structure of TF as described by Ruf et al., Proc. Natl. Acad. Sci., USA, 88:8430-8434 (1991) in conjunction with the mutations of residues 151-174 of this invention are consistent with this hypothesized immunoglobulin-like fold of TF. Residues Tyr^{157}, Lys^{159}, Ser^{163}, Gly^{164}, Lys^{165} and Lys^{166} were identified as important for function, either directly or indirectly through maintenance of a functional structure in the predicted 157-167 loop. Replacement of Gly^{164} with the larger and more rigid Ala resulted in severe loss of function consistent with location of Gly^{164} in a turn which may be required for the proper conformation of the putative 157-167 loop. All dysfunctional mutants in the 157-167 region were characterized by high affinity binding of VII/VIIa and the ability to form mutant TF-VIIa complexes which efficiently hydrolyzed small peptidyl substrates. These data are consistent with expression of mutant proteins with proper overall fold.

The functionally defective mutants displayed two phenotypes. Whereas removal of the Lys^{159} side chain only affected the conversion of VII to VIIa, the other mutants formed catalytic binary complexes with selectively reduced proteolytic activity for Factor X and a suggested consecutive defect in VII activation.

The Tyr^{157} to Ala mutation in the amino-terminal aspect of the predicted loop resulted in a functional

phenotype similar to the charge modifying mutations in the carboxyl aspect. It must be considered whether Tyr^{157} may be important for stabilization of the loop by providing a hydrophobic center with its aromatic side chain. Consistent with this possibility, the Tyr^{157} to Phe substitution has been shown to be compatible with full functional activity of TF as described by Rehemtulla et al., Biochem J., 282:737-740 (1992). A supporting role for the structure of the 157-167 loop can also be considered for residues Ser^{162} and Ser^{163}. Although functionally important interactions for the Ser^{163} side chain cannot be excluded, loss of function due to side chain capping (Ser^{163}) or introduction of additional side chain atoms (Ser^{162}) would also be consistent with a perturbation of the structural integrity of the 157-167 loop resulting in loss of proper alignment of adjacent functionally important residues.

Thus, a discrete structure in TF is required for efficient hydrolysis of protein substrates. This function may be mediated through specific contacts with VIIa which contribute minimally to the binding energy. Alternatively, this region in TF may form direct contacts with the protein substrates contributing significantly to extended protein substrate recognition. The present evidence is consistent with a model of an interactive surface on the TF-VIIa complex for protein substrates which is composed entirely or in part by TF residues. It appears that these residues contribute differently to the activation of X and VII. The human TF mutants characterized here thus help to define the molecular structures which mediate the well documented roles of

TF as an enhancer of VII activation as described by Nakagaki et al., Biochem., 30:10819-10824 (1991) and Rao et al., Proc. Natl. Acad. Sci., USA, 85:6687-6691 (1988) and as a catalytic cofactor for specific proteolysis of protein substrates X and IX as described by Ruf et al., J. Biol. Chem., 266:2158-2166 (1991), Silverberg et al., J. Biol. Chem., 252:8481-8488 (1977) and Lawson et al., J. Biol. Chem., 266:11317-11327 (1991) which trigger the subsequent proteolytic events in the coagulation cascade.

5. Reconstitution of Purified Tissue Factor Mutants with Phospholipid

A. Preparation of Phospholipids

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG) are obtained in chloroform solution from Avanti Polar Lipids in Alabaster, Alabama, or Calbiochem Corporation in La Jolla, California, in sealed glass ampules and stored under N_2 at -20C. CHAPS, other detergents and bovine gamma globulin were obtained from Calbiochem. Tris base and glycine are purchased from BioRad Laboratories in Richmond, California. All other chemicals and biochemicals are acquired from Sigma in St. Louis, Missouri.

Phospholipids are prepared for resolubilization in the following manner. PC, PE, PS, and PG are warmed to room temperature and combined in a suitable tube or flask at the specified mole ratios. The antioxidant, butyrated hydroxytoluene (BHT), is dissolved in chloroform and added to the mixture of

phospholipids at a weight ratio of 0.1% (BHT:total phospholipids). Organic solvent is removed by evaporation under a stream of dry nitrogen or under reduced pressure in a rotary evaporator. Residual organic solvent is eliminated by pumping an additional 1 hour at room temperature with a vacuum pump at a pressure of 10 mm or less. The mixture of phospholipids is redissolved to 20 mg/ml in 20 mM Tris-HCl at pH 7.5, 150 mM NaCl (TBS) containing 100 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate).

The tissue factor mutants prepared in Example 2 are then separately admixed with carrier protein and are then combined with the redissolved phospholipids prepared above. The volume of the resulting mixture is adjusted with a buffer as described above, preferably containing cryopreservative (most preferably trehalose) and glycine but no detergent. Various permutations for the preparation of phospholipid-mutant TF reagents of this invention are presented below.

B. Preparation of the Mutant TF/Phospholipid (mhuTF/P) Reagents by Dialysis

Phospholipids are combined at the specified mole ratios of PC, PE, PS, and PG, then resolubilized as described above. The resolubilized phospholipids are combined with the mutant TF proteins of this invention and bovine gamma globulin. Additional TBS containing 150 mM trehalose is added to yield final concentrations of 4 mg/ml total phospholipid, 10 mg/ml mhuTF, 1 mg/ml bovine gamma globulin and 20 mM CHAPS.

This clear and colorless solution is placed in a dialysis membrane tubing (Spectrapore®, Spectrum Medical Industries, molecular weight cutoff of 12,000 to 14,000) and dialyzed for at least 30 hours at room temperature against TBS containing 150 mM trehalose and 0.05% NaN_3. After dialysis the volume of the dialysate is determined and adjusted back to the original volume, if required, with dialysis buffer. $CdCl_2$ is added to a final concentration of 5 mM and the solution is incubated at 37C for 2 hours.

The solution is frozen on dry ice, then lyophilized using a cycle beginning at -40C and ending at room temperature, over a 48 hour period. The liposomes are then reconstituted to a working concentration with 0.1 M Tris-HCl at pH 7.5, 150 mM trehalose to yield a solution containing mhuTF at approximately 1 to 2 mg/ml, phospholipids at approximately 400 to 800 mg/ml, and bovine gamma globulin at 50 to 100 mg/ml.

C. Preparation of Preparation of the Mutant TF/Phospholipid (mhuTF/P) Reagents Without Dialysis

Phospholipids are prepared for resolubilization in the following manner. PC, PE, and PS are warmed to room temperature and combined in a suitable tube or flask at a mole ratio of 7.5:1:1 of PC, PE, and PS, respectively. The antioxidant, butyrated hydroxytoluene (BHT), is dissolved in chloroform and added to the mixture of phospholipids at a weight ratio of 0.1% (BHT:total phospholipids). Organic solvent is removed by evaporation under a stream of dry nitrogen or under reduced pressure in a

rotary evaporator. Residual organic solvent is eliminated by pumping an additional 1 hour at room temperature with a vacuum pump at a pressure of 10 mm or less.

The mixture of phospholipids is redissolved in 50 mM octyl beta-D-thioglucopyranoside (OTG) in 20 mM HEPES (pH 6), 150 mM NaCl to a final concentration of 4 mg/ml. The mutant TF (mhuTF) proteins from Example 2 and bovine gamma globulin are mixed with the resolubilized phospholipids. Enough 20 mM HEPES (pH 6), 150 mM NaCl is added to adjust the final concentrations to 10 mg/ml mhuTF, 1 mg/ml bovine gamma globulin, 4 mg/ml phospholipids, and 10 mM OTG. $CdCl_2$ is added to a final concentration of 5 mM to activate the mhuTF. The resulting mixed micelles comprised of mhuTF, OTG, and phospholipids are diluted with 20 mM HEPES, pH 6, 150 mM NaCl to yield a solution containing mhuTF at approximately 0.5 to 1 mg/ml, phospholipids at approximately 500 to 700 mg/ml, and bovine gamma globulin at 25 to 50 mg/ml to give mhuTF PT reagent.

D. Preparation of Mutant TF/Phospholipid (mhuTF/P) Reagent by Diafiltration

Phospholipids are combined at mole ratio of 7.5: 1: 1 (PC: PE: PS), dried to remove organic solvent, then resolubilized as described above. The resolubilized phospholipids at 15 mg/ml in TBS containing 100 mM CHAPS are combined with mutant TF proteins prepared in Example 2 and bovine gamma globulin. Additional TBS containing 150 mM trehalose is added to yield final concentrations of 4 mg/ml phospholipid, 10 mg/ml mhuTF, 1 mg/ml bovine gamma globulin and 20 mM CHAPS.

The detergent (CHAPS) is removed by tangential flow diafiltration using, a Pyrostart or Ultrastart filter unit (Sartorius Corp., Bohemia, NY, molecular weight cutoff of 20,000) and TBS containing 150 mM trehalose as the dialysis buffer. Approximately 95 to 100% of the CHAPS can be removed by passing 10 volumes of dialysis buffer through the device. After diafiltration the volume of the dialysate is determined and adjusted back to the original volume (if required) with TBS containing 150 mM trehalose and 0.05% NaN_3. $CdCl_2$ is added to a final concentration of 5 mM and the solution was incubated at 37C for 2 hours.

The solution may be frozen on dry ice, then lyophilized using a cycle beginning at -40C and ending at room temperature, over a 48 hour period. The resulting reagent may be reconstituted to working concentration with the addition of 0.1 M Tris-HCl at pH 7.5, 150 mM trehalose to yield a solution containing mhuTF at approximately 1 to 2 mg/ml, phospholipids at approximately 400 to 800 mg/ml, and bovine gamma globulin at 50 to 100 mg/ml.

E. Preparation of Mutant TF/Phospholipid (mhuTF/P) Reagent by Addition of XAD-2 Resin

Phospholipids are combined at mole ratio of 67: 16: 10: 7 (PC: PG: PE: PS), dried to remove organic solvent, then resolubilized as described above. The resolubilized phospholipids at 15 mg/ml in TBS containing 100 mM CHAPS and 0.8% glycine are combined mutant TF proteins prepared in Example 2 and bovine gamma globulin. Additional TBS containing 150 mM trehalose and 0.8% glycine is added to yield final

concentrations of 3 mg/ml phospholipid, 4.5 mg/ml mhuTF, 1 mg/ml bovine gamma globulin and 20 mM CHAPS.

Hydrophobic chromatographic resins such as Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa) or Bio-Beads SM-2 (BioRad, Richmond, Ca) can also be used to remove the detergent (CHAPS), either in direct contact with the phospholipid solution or separated from it by a dialysis membrane. The rate of removal is proportional to the weight ratio of the detergent in solution and the chromatographic resin beads. Indeed, the rate of removal is proportional to both the amount of resin added and the rate of addition. The amount required to remove all of the detergent is calculated from the capacity of the resin (provided by the manufacturer) and the total mass of detergent to be removed. Moreover, 99.9% removal of the detergent may be achieved either in 1 hour or in 24 hours, at 30C depending upon the rate at which this amount of resin is added. $CdCl_2$ was added to a final concentration of 5 mM and the solution was incubated at 37C for 2 hours. The liposomes are then diluted to a working concentration with 50 mM Tris-HCl at pH 7.5, 75 mM trehalose, 15 mM $CaCl_2$, 0.8% glycine, 1% maltose, and 0.05% NaN_3 to yield a solution containing mhuTF at approximately 0.04 to 0.20 mg/ml, phospholipids at approximately 40 to 150 mg/ml, and bovine gamma globulin at 50 to 100 mg/ml.

The solution is frozen on dry ice, then lyophilized using a cycle beginning at -40C and ending at room temperature, over a 48 hour period. The lyophilized reagent was reconstituted with distilled water prior to use.

Following the above preparations, the resultant

phospholipid-reconstituted tissue factor mutants of this invention can then be used in clinical clotting assays where the accurate determination of the concentration of plasma factor VIIa is required without the confounding of the amount of VIIa produced by the rapid conversion of VII to VIIa by normal tissue factor.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: METHODS FOR ASSAYING FACTOR VIIa USING MUTANT RECOMBINANT TISSUE FACTOR LACKING FACTOR VII ACTIVATION ACTIVITY, AND COMPOSITIONS THEREFOR

(iii) NUMBER OF SEQUENCES: 26

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US93
(B) FILING DATE: 06-OCT-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/957,985
(B) FILING DATE: 06-OCT-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5437 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 2267..2362

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2363..3154

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AATACTGTCC TTCTAGTGTA GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG 180

CCTACATACC TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG 240

TGTCTTACCG GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG GTCGGGCTGA 300

ACGGGGGGTT CGTGCACAGA GCCCAGCTTG GAGCGAACGA CCTACACCGA ACTGAGATAC 360

CTACAGCGTG AGCATTGAGA AAGCGCCACG CTTCCCGAAG GGAGAAAGGC GGACAGGTAT 420

CCGGTAAGCG GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC 480

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AGAAATTGTA AACGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC 660

ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGCCCGA 720

GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA CTATTAAAGA ACGTGGACTC 780

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                          Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr
                            1               5                  10

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Asn Leu Thr Trp Lys Ser Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu
                  15                  20                  25

CCC AAA CCC GTC AAT CAA GTC TAC ACT GTT CAA ATA AGC ACT AAG TCA      2488
Pro Lys Pro Val Asn Gln Val Tyr Thr Val Gln Ile Ser Thr Lys Ser
             30                  35                  40

GGA GAT TGG AAA AGC AAA TGC TTT TAC ACA ACA GAC ACA GAG TGT GAC      2536
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GTC TTC TCC TAC CCG GCA GGG AAT GTG GAG AGC ACC GGT TCT GCT GGG      2632
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                255                 260
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TCGTTGTGTA GGTACCGCTG TATTCCTAGG GAAATAGTAG AGGCACCTTG AACTGTCTGC 4541

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TACACCTCCT CTGAAATACC CATAGTTGCT AGGGCTGTCT CCGAACTCAT TACACGCTAC 4661

CAAGTGAGAG CTGTAATTTC GCGATCAAGG GCAGCGAGGG CTTCTCCAGA TAAAATAGCT 4721

TCTGCCGAGA GTCCCGTAAG GGTAGACACT TCAGCTAATC CCTCGATGAG GTCTACTAGA 4781

ATAGTCAGTG CGGCTCCCAT TTTGAAAATT CACTTACTTG ATCAGCTTCA GAAGATGGGC 4841

GAGGGCCTCC AACACAGTAA TTTTCCTCCC GACTCTTAAA ATAGAAAATG TCAAGTCAGT 4901

TAAGGAGGAA GTGGACTAAC TGACGGACCT GGCCGTGCGA CATCCTCTTT TAATTAGTTG 4961

CTAGGCAACG CCCTCCAGAG GGCGTGTGGT TTTGCAAGAG GAAGCAAAAG CCTCTCCACC 5021

CAGGCCTAGA ATGTTTCCAC CCAATCATTA CTATGACAAC AGCTGTTTTT TTTAGTATTA 5081

AGCAGAGGCC GGGGACCCCT GGGCCCGCTT ACTCTGGAGA AAAAGAAGAG AGGCATTGTA 5141

GAGGCTTCCA GAGGCAACTT GTCAAAACAG GACTGCTTCT ATTTCTGTCA CACTGTCTGG 5201

CCCTGTCACA AGGTCCAGCA CCTCCATACC CCCTTTAATA AGCAGTTTGG GAACGGGTGC 5261

GGGTCTTACT CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA 5321

TCGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT CTGAGCTATT 5381

CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGCTTT TGCAAAAAGC TAATTC 5437

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
1 5 10 15

Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
20 25 30

Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
35 40 45

Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
50 55 60

Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
65 70 75 80

Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn

85 90 95

Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
100 105 110

Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
115 120 125

Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
130 135 140

Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
145 150 155 160

Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
165 170 175

Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
180 185 190

Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
195 200 205

Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu Ile Phe Tyr Ile Ile
210 215 220

Gly Ala Val Val Phe Val Val Ile Ile Leu Val Ile Ile Leu Ala Ile
225 230 235 240

Ser Leu His Lys Cys Arg Lys Ala Gly Val Gly Gln Ser Trp Lys Glu
245 250 255

Asn Ser Pro Leu Asn Val Ser
260

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATACACTTT ACGCGTGGGC ATCTTCAAGT 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGGAAATCT GCAGATGCAG GAAAGAAA 28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAAATCTGC CTCGAGTGGA AAGAA 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATCTTCAGC CTCGGGAAAG AA 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGAAATCCT CGAGTGCAGG AAA 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TATTGGAAAT CCTCGAGTTC AGCAAAGAAA ACA 33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCTTCAAGCT CAGGAAAGAA AGCAGCCAAA 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGGAAATCTT CAACCTCGGG AAAGAAA 27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGGAAATCTT CAGACTCGGG AAAGAAA 27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGGAAATCTT CAAACTCGGG AAAGAAA 27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGCAAGGAC GCGATCGCTA CAGCTTATTA TTGG 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAAACAGCTG CAACAGCCAC TGCTGAGTTT 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCCAAAGCAA ACGCTAATGC GTTTTTGATC GATGTG 36

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTTTATTATC GAAAAGGTTC AAGTTCA 27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TATTATTGGG CATCCTCGAG TTCAGGA 27

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTTTATTATC GCGCAGGTTC AAGTTC 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCAAGGACC TCGGGTATAT ACTTACTTAT TGGAAA 36

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCAAGGACC TCGGGTATAT ACTTACTTAT CGGAAA 36

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATACACTTT ACGCGTGGAA ATCT 24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TGTTTTTGGC GCAGCTTTAA TTTATA 26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTGGAAATCT GCAGATGCAG GAAAGAAA 28

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAGTTCAGGC GCGGCAACAG CCAAAA 26

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CGGGATGTAG CTGGCAAGG 19

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATGAGTTTGC GATCGCTGTG GCTAAAGGAG 30

What Is Claimed Is:

1. A method for detecting the amount of Factor VIIa in a body fluid sample comprising the steps of:

a) admixing a preselected amount of said body fluid sample with a clotting assay admixture, wherein said clotting assay admixture comprises a composition containing mutant human tissue factor protein (mhuTF) that is substantially free of Factor VIIa and wild type human tissue factor, to form a Factor VIIa assay admixture, wherein said mhuTF has the capacity to bind Factor VII/VIIa and to proteolytically hydrolyze Factor X when present in a complex of mhuTF:VIIa, but being substantially free of the capacity to activate Factor VII when present in a complex of mhuTF:VIIa;

b) maintaining said Factor VIIa assay admixture under conditions sufficient for said mhuTF to bind to any of said Factor VIIa in said sample and form a clot; and

c) determining the amount of time required for the clot to form, which time is proportional to a predefined amount of Factor VIIa, thereby determining the amount of VIIa present in said sample.

2. The method of claim 1 wherein said mutant human tissue factor protein comprises at least one amino acid residue substitution in the region of wild type human tissue factor defined by residues 106 to 219 of the sequence shown in SEQ ID NO 2.

3. The method of claim 2 wherein said substitution is selected from the group consisting of $R^{158}G^{160}$, A^{159}, $R^{158}A^{159}G^{160}$, A^{157}, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$.

4. The method of claim 2 wherein said mutant

human tissue factor protein has an amino acid residue sequence consisting of the sequence shown in SEQ ID NO 2 with a substitution selected from the group consisting of $R^{158}G^{160}$, A^{159}, $R^{158}A^{159}G^{160}$, A^{157}, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$.

5. The method of claim 1 wherein said composition further comprises liposomes.

6. The method of claim 5 wherein said composition comprises:

(a) a phospholipid mixture comprising:

(i) from about 20 to about 95 mole percent phosphatidylcholine;

(ii) from about 2.5 to about 50 mole percent phosphatidylethanolamine;

(iii) from about 2.5 to about 50 mole percent phosphatidylserine;

(iv) from about 0 to 40 mole percent phosphatidylglycerol; and

(b) from about 0.1 mg to about 3 mg of mhuTF per mg phospholipid mixture.

7. The method of claim 5 wherein said composition further comprises from about 0.5 percent to about 1.5 percent glycine.

8. The method of claim 5 wherein said composition further comprises a carbohydrate cryopreservative selected from a group consisting of trehalose, maltose, lactose, glucose, and mannitol.

9. The method of claim 5 wherein said composition further comprises a detergent.

10. The method of claim 9 wherein said detergent is an alkyl glucopyranoside.

11. The method of claim 10 wherein said detergent is selected from the group consisting of

octyl beta-D-glucopyranoside and octyl beta-D-thioglucopyranoside.

12. A mutant human tissue factor protein (mhuTF) having the capacity to bind Factor VII/VIIa and to proteolytically hydrolyze Factor X when present in a complex of mhuTF:VIIa, but being substantially free of the capacity to activate Factor VII when present in a complex of mhuTF:VIIa, wherein said mhuTF protein comprises at least one amino acid residue substitution in the region of wild type human tissue factor defined by residues 106 to 219 of the sequence shown in SEQ ID NO 2, but not the substitutions $R^{158}G^{160}$, A^{159} or A^{157}.

13. The mutant human tissue factor of claim 12 wherein said substitution is selected from the group consisting of $R^{158}A^{159}G^{160}$, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$.

14. The mutant human tissue factor of claim 12 wherein said protein has an amino acid residue sequence consisting of the sequence shown in SEQ ID NO 2 with a substitution selected from the group consisting of $R^{158}A^{159}G^{160}$, $A^{161}D^{162}A^{163}$, $A^{165}A^{166}$, A^{147}, and $G^{152}I^{154}T^{156}R^{158}G^{160}$.

15. A composition containing a mutant human tissue factor protein according to claim 12.

16. The composition of claim 15 further comprising liposomes.

17. The composition of claim 16 comprising:

(a) a phospholipid mixture comprising:

(i) from about 20 to about 95 mole percent phosphatidylcholine;

(ii) from about 2.5 to about 50 mole percent phosphatidylethanolamine;

(iii) from about 2.5 to about 50

mole percent phosphatidylserine;

(iv) from about 0 to 40 mole percent phosphatidylglycerol; and

(b) from about 0.1 mg to about 3 mg of mhuTF per mg phospholipid mixture.

18. The composition of claim 16 further comprising from about 0.5 percent to about 1.5 percent glycine.

19. The composition of claim 16 further comprising a carbohydrate cryopreservative selected from a group consisting of trehalose, maltose, lactose, glucose, and mannitol.

20. The composition of claim 16 further comprising a detergent.

21. The composition of claim 20 wherein said detergent is an alkyl glucopyranoside.

22. The composition of claim 21 wherein said detergent is selected from the group consisting of octyl beta-D-glucopyranoside and octyl beta-D-thioglucopyranoside.

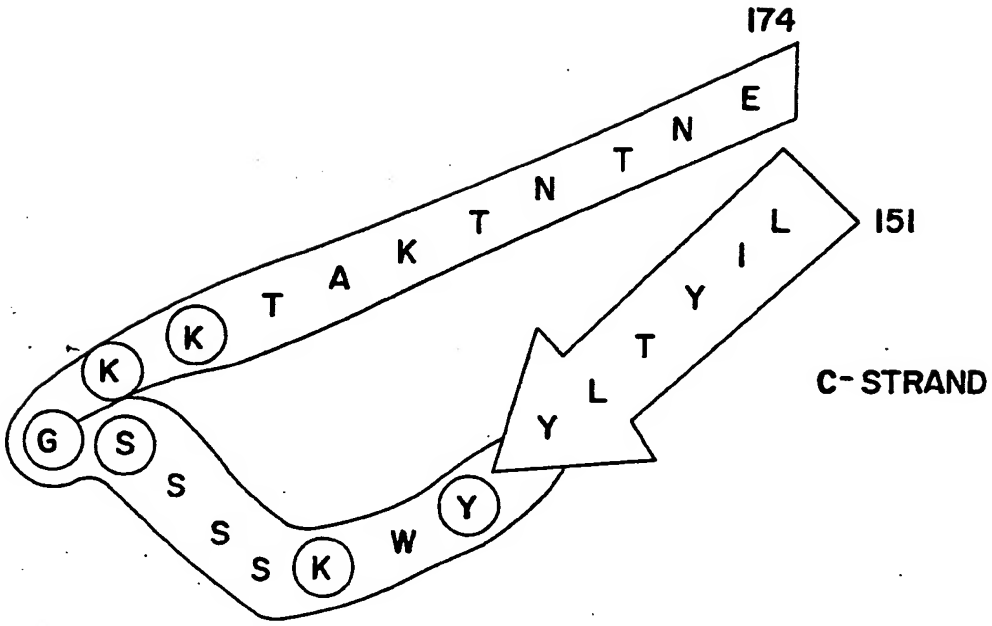

FIG. 1

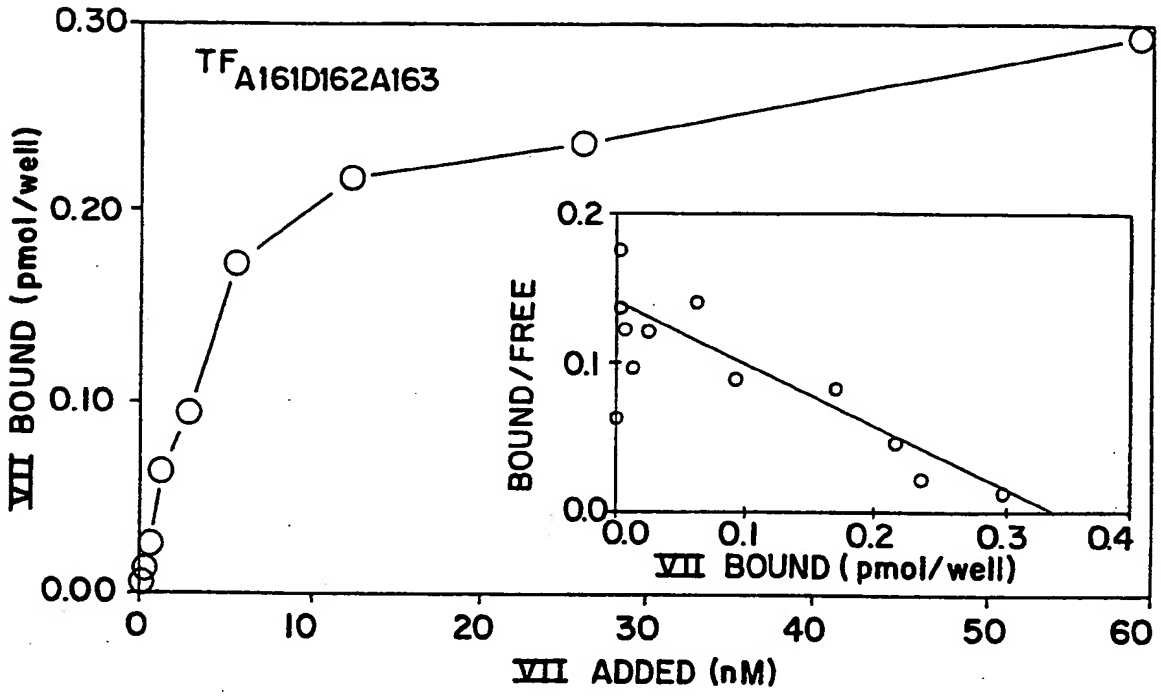
TF A161D162A163
VII BOUND (pmol/well)
VII ADDED (nM)
BOUND/FREE
VII BOUND (pmol/well)

FIG.2A

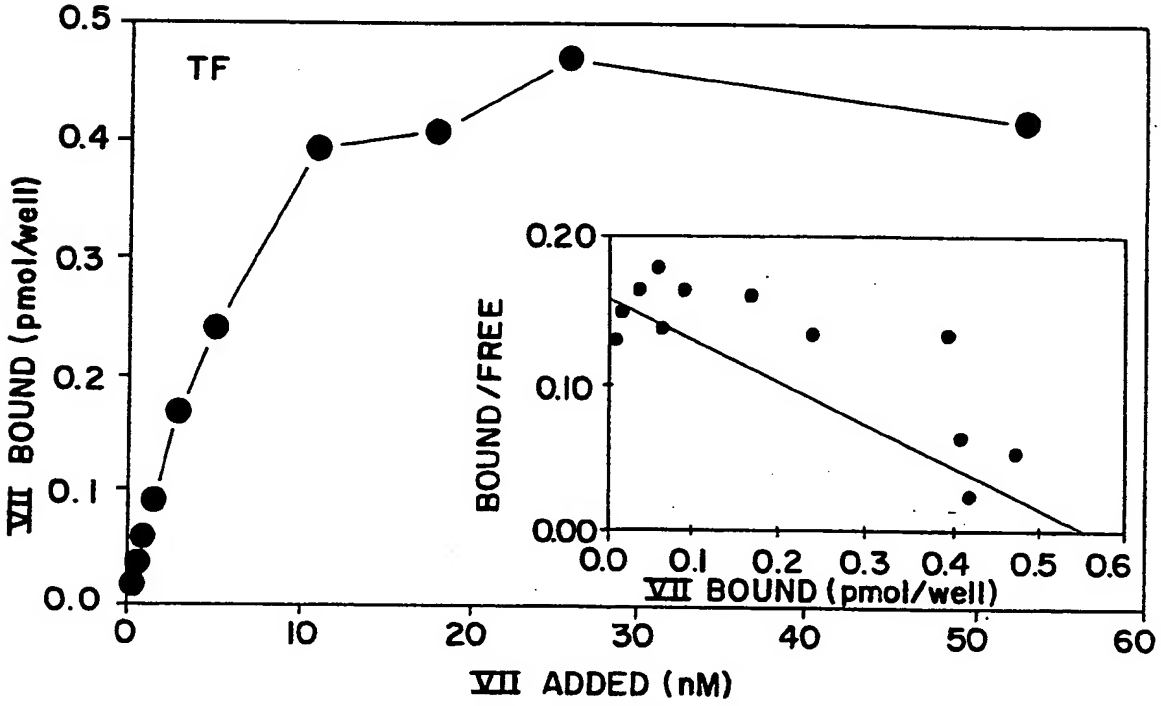
TF
VII BOUND (pmol/well)
VII ADDED (nM)
BOUND/FREE
VII BOUND (pmol/well)

FIG.2B

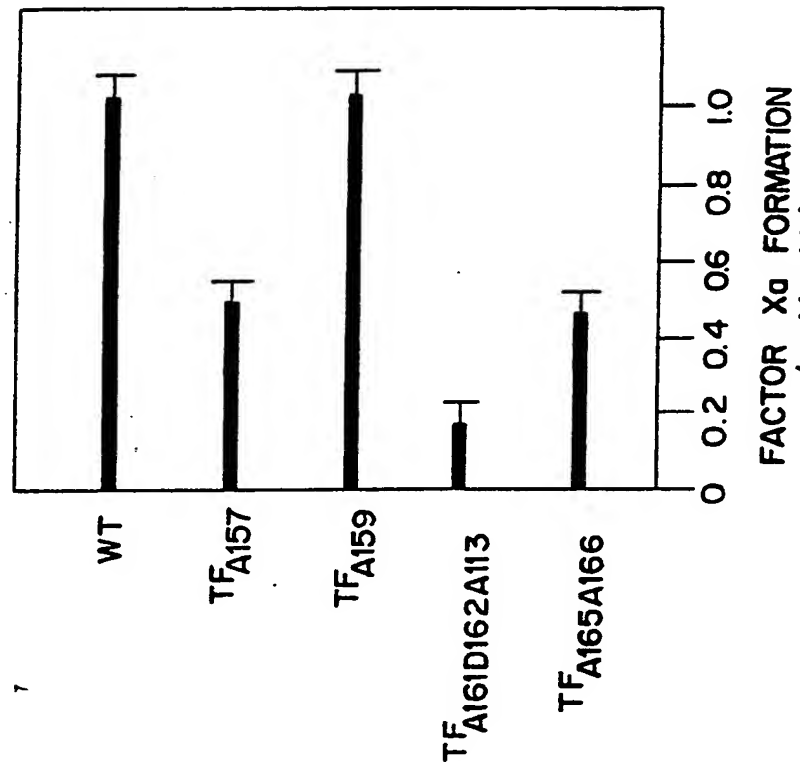
FACTOR Xa FORMATION
(mol/mol/s)
0
0.2
0.4
0.6
0.8
1.0
WT
TF A157
TF A159
TF A161D162A113
TF A165A166

FIG.3B

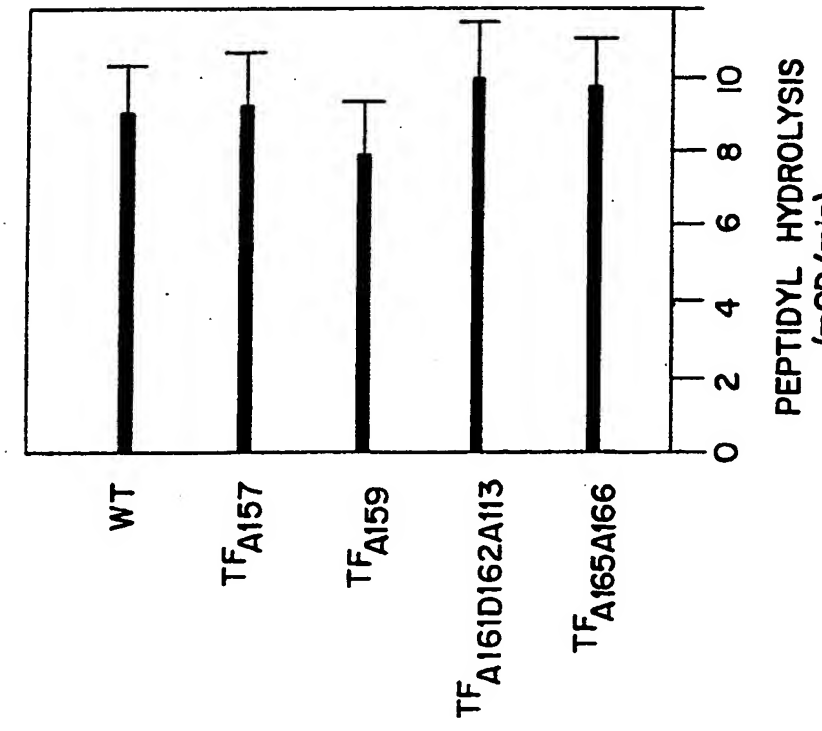
PEPTIDYL HYDROLYSIS
(mOD/min)
0
2
4
6
8
10
WT
TF A157
TF A159
TF A161D162A113
TF A165A166

FIG.3A

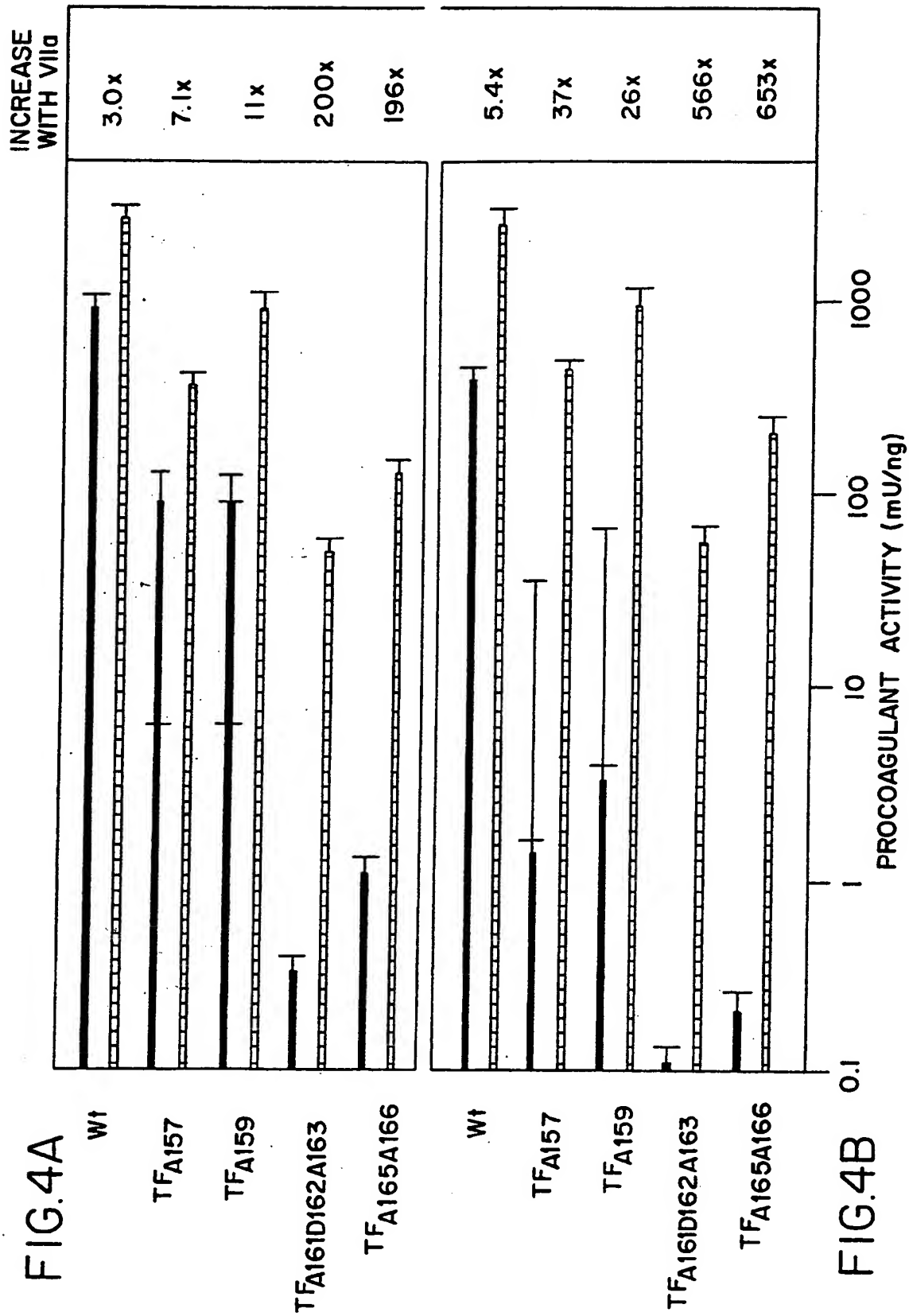
INCREASE WITH VIIa
3.0x
7.1x
11x
200x
196x
5.4x
37x
26x
566x
653x
Wt
TFA157
TFA159
TFA161D162A163
TFA165A166
Wt
TFA157
TFA159
TFA161D162A163
TFA165A166
0.1
1
10
100
1000
PROCOAGULANT ACTIVITY (mU/ng)

FIG.4A

FIG.4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09570

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00, 37/54; C07K 13/00; G01N 33/86
US CL :424/94.3, 94.63; 514/8, 12; 435/4, 13, 212, 226; 530/380, 381
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.3, 94.63; 514/8, 12; 435/4, 13, 212, 226; 530/380, 381

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Online, MEDLINE, APS, World Patents Index

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO, A, 92/18870 (Morrissey et al) 29 October 1992, see entire document.	1-22
Y	Biochemical Journal, Volume 282, issued 15 March 1992, A. Rehemtulla et al, "The Third Trp-Lys-Ser (WKS) Tripeptide Motif in Tissue Factor is Associated with a Function Site", pages 737-740, see entire document.	1-22
Y	The Journal of Biological Chemistry, Volume 266, No. 4, issued 05 February 1991, W. Ruf et al, "Phospholipid-independent and -dependent Interactions Required for Tissue Factor Receptor and Cofactor Function", pages 2158-2166, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 December 1993	Date of mailing of the international search report 05 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer R. KEITH BAKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09570

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Biological Chemistry, Volume 267, No. 4, issued 25 March 1992, W. Ruf et al, "Cofactor Residues Lysine 165 and 166 are Critical for Substrate Regognition by the Tissue Factor-Factor VIIa Protease Complex", pages 6375-6381, see entire document.	1-22
Y	The Journal of Biological Chemistry, Volume 266, No. 32, issued 15 November 1991, S. Roy et al, "Lysine Residues 165 and 166 are Essential for the Cofactor Function of Tissue Factor", pages 22063-22066, see entire document.	1-22
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